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UMI

Pesticide Induced Immunosuppression in Northern Leopard Frogs (*Rana pipiens*)

By
Mary-Kate Gilbertson

A Thesis
Submitted to the Faculty of Graduate Studies and Research
Through the Great Lakes Institute for Environmental Research
and
The Department of Biological Sciences
in Partial Fulfillment of the Requirements
for the Degree of

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ABSTRACT

This thesis investigated the hypothesis that environmental xenoibiotics have the potential to alter the immune function of a variety of anuran species. Specifically, the effects of pesticides on the immune function of the northern leopard frog (*Rana pipiens*) were investigated. Three assays, IgM specific antibody response to KLH-DNP, zymozan induced chemiluminescence of whole blood (CL) and the delayed type hypersensitivity (DTH), were used to assay humoral, innate and cell-mediated immune endpoints. To determine whether pesticides had a measurable effect on the anuran immune system, sublethal doses of *o,p* DDT (750 ng/g w.w.), malathion (330 ng/g w.w.) and dieldrin (75 ng/g w.w.) were injected into adult leopard frogs. The antibody response was dramatically suppressed compared to the control group in all pesticide injected groups with recovery of this response occurring in all frogs between eight and twenty weeks following exposure. The DTH reaction at two weeks after pesticide treatment, was enhanced and the respiratory burst was lower in the pesticide groups. This study indicated that sublethal exposure to some pesticides measurably altered some immune parameters in adult leopard frogs. A dose response study using DDT (0.075-75 ng/g w.w) revealed that at lower doses, the pesticide continued to suppress the antibody response, although the CL response and the DTH were enhanced. This study confirmed that the assays could detect alterations in immune response caused by DDT exposure and that at lower doses some parameters were suppressed while others were enhanced. When the order of immunisation was reversed, no alteration in the immune system was apparent between the control and dosed groups, indicating that if a frog population was exposed to

an immunological challenge prior to pesticide exposure, they might still be capable of mounting a response.

An Ontario field study, found both stimulatory and suppressive effects in frogs from Essex county when compared to those collected near Collingwood. The antibody response and CL were suppressed, and the DTH enhanced in Essex county frogs. Point Pelee however showed an elevated CL response. Very high levels of contaminants were found in one frog from Point Pelee and organochlorines were found to be elevated in Essex in comparison to Ottawa or Collingwood.

Overall, the results suggest that some organochlorine and organophosphate pesticides do alter the immune system of adult leopard frogs. Depending upon many factors such as stress, dose and time since exposure, these results may be stimulatory or suppressive. More conclusive statements regarding the immunological status of different populations might be obtained by an *in situ* cage study.

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unfailing support, I am truly blessed with good genetics. Lastly, I would like to thank the frogs that have participated in these experiments and express the hope that the information attained might contribute in some way to making the future brighter for their ranid relatives.

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ABBREVIATIONS

ANOVA - analysis of variance
BSA - bovine serum albumin
CL - chemiluminescence assay
Con A - concanavlin A
Cy - cyclophosphamide
DCM - dichloromethane
DDA - dimethyldioctadecylammonium bromide
DDD - 1,1-dichloro-2,2'-bis(*p*-chlorophenyl)ethane
DDE - 1,1-dichloro-2,2'-bis(*p*-chlorophenyl)ethylene
DDT – 1,1-dichloro-2,2'-diphenyltrichlorethane
DMSO - dimethyl sulphoxide
DTH - delayed-type hypersensitivity reaction
ELISA - the enzyme-linked immunosorbent assay
FITC - fluorescein isothiocyanate
GLIER - Great Lakes Institute of Environmental Research
KLH - keyhole limpet haemocyanin
LC_{50} - lethal concentration
LOAEL - lowest observed acute effect level
LPS - lipopolysaccharide
Ig - immunoglobulin
IgM - immunoglobulin M
nm - nanometers
OC - organochlorine
OOS-TMP - O,O,S,-trimethyl phosphorothioate
OP - organophasphate
PAHs - polycyclic aromatic hydrocarbons
PBS - phosphate buffered saline
PHA - phytohemagglutinin
PWM - pokeweed mitogen
SRBCs - anti-sheep red blood cells
TBS-T – tris-buffered saline solution with tween
UV-B - ultraviolet B radiation

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THESIS AIMS AND OBJECTIVES

The aim of this project was to develop functional immune assays applicable to amphibians so that species specific immune endpoints can be examined. The intention was to develop non-sacrificial methods, applicable in a field situation.

The objective was:

1) To develop immunological assays to determine whether certain pesticides at known concentrations altered the immune function of one species of Ontario frog, the northern leopard frog.

2) To apply these assays to frogs, collected from various regions of Ontario to determine whether immune function could be correlated to tissue contaminant concentrations.

CHAPTER 1.0 – INTRODUCTION

1.1 Amphibian Declines

Amphibians have existed for over 350 million years during which time they have suffered extinctions of some groups and emergence of others [1]. Historically, unprecedented changes in amphibian numbers have been observed over the past 25 years [1, 2]], and on every continent, attention has turned to threats to the long-term survival and size of amphibian populations [3]. Losses of amphibian species and populations are of global concern [4-8], because frogs not only consume insect pests [9, 10] and are major contributors to community biomass [9-11] but also play significant roles in competitive and predator-prey relationships [11].

In some cases, a variety of kinds of human disruption is strongly implicated with these declines because of the size of geographical areas affected and the pace with which these declines are occurring. In other cases no suspected cause or causes have been hypothesized [12]. Numerous hypotheses have been suggested to explain the losses such as; pollution [1, 13, 14]; habitat modification and degradation [13, 14]; over collection by suppliers [13, 14]; increased prevalence of disease [1, 13, 14]; introduction of bullfrogs [14, 15]; introduction of exotic fish as predators and competitors [14] and increased ultraviolet B radiation (with and without interaction by chemicals)[2, 6, 14]. The available evidence suggests that more than one agent is contributing to amphibian declines [2].

1.2 Amphibians as Biomonitors and Bioindicators

Many reviewers of these amphibian declines suggested, for the following reasons, that amphibians are reliable indicators of the accumulation and effects of a variety of contaminants. The ecology and physiology of amphibians expose them to a wide variety of routes of contamination [16]; The thinness and enhanced permeability of amphibian skin, which is necessary for respiration, make them more exposed to waterborne and airborne toxicants than are other vertebrates [12, 16-18]; Amphibians are mainly herbivorous as tadpoles and insectivores as adults, and some have complex life cycles which involve both an aquatic and terrestrial phase, potentially exposing them to terrestrial and aquatic xenobiotics [16, 19]; Amphibians hibernate in soil and mud, potentially exposing them to toxic conditions [20]; Amphibians are more resistant to some pesticides than are other biological groups, and they accumulate them in quantities that are potentially toxic to their predators [21]; Amphibians typically have a small home range and are relatively long lived; They frequently live in small, temporary ponds and forested areas, which are often targets of aerial pesticide sprays [22, 23], agricultural field or urban runoff, [24], industrial chemicals [25], and nutrients [3, 26]. These characteristics not only make them suitable as candidate bioindicators, but also result in excellent model systems to develop *in situ* cause and effect relationships for toxic chemicals [27].

1.3 Amphibian Pesticide Residues Levels

Pesticides are xenobiotics that are deliberately added to the environment for the purpose of killing a form of life [28]. Initially the use of organochlorine (OCs) chemicals was widespread, however since the early 1970s they have been restricted or banned altogether. Although they are no longer in use, many OCs continue to cause effects on a variety of organisms in the environment because of their extremely long half-life in soil and their bioaccumulation in food chains. These chemicals can enter groundwater supplies and have a propensity to be concentrated in adipose tissue and thus enter the food chain [28].

Organochlorine (OCs) pesticides [20, 29-32] have been measured in whole body analysis of frogs and toads. Studies across North America have documented the presence of a variety of compounds in amphibian tissue. The information is however sparse, as amphibians have never been used routinely as biomonitors [33]. Historical and recent analysis of OC residues in a number of species in Ontario and further afield are shown in Appendix A.

One study in 1974 showed that high levels of pesticides detected in frogs after a pesticide spraying event. Analysis showed extremely high tissue concentrations of dichlorodiphenyltrichlorethane (DDT) in western spotted frogs (*Rana pretiosa*), at a site in Oregon, following aerial spraying [34]. Dead frogs collected three weeks after the spraying had up to 6670 ng/g 1,1-dichloro-2,2'-bis(*p*-chlorophenyl)ethane (DDD), 366 ng/g of 1,1-dichloro-2,2'-bis(*p*-chlorophenyl)ethylene (DDE) and 5670 ng/g DDT in whole body extracts and live frogs collected had 403 ng/g DDD, 173 ng/g DDE and 1750 ng/g DDT [34].

Another study of DDT exposure in New Brunswick showed that exposure to the pesticide did effect frog populations. Heavy doses of DDT, which was sprayed in a 120 ha area of a forest in 1952 led to considerable mortality of larval amphibians [35].

This historical pesticide use continues to impact amphibians today. OC residues in amphibians from Ontario show evidence of persistence and presence in areas thought of as refuges. Russell *et al.*, found that in Point Pelee National Park, Ontario, spring peepers *(Pseudacris crucifer)* retained high levels of DDT and metabolites 26 years after spraying in the park with DDT for mosquito control had abated [36]. This suggests that an unknown point source may still exist within the park. Levels of *p'p,*-DDE in tissues were found to be greater than 1000 µg/kg. In another study involving several sites in Ontario, where OC insecticides had been banned for more than 25 years [32], variable concentrations of *p,p'*-DDE (0.58-45 ng/g) were found in the tissue of green frogs (*Rana clamitans*). The DDE concentrations in this study tended to be highest in agricultural areas and indicated that contaminant accumulation in green frogs tended to be site specific [32].

Conversely, a study examining both green and leopard frogs from different regions of southern Ontario found site to site variability in chemical concentrations which tended to reflect local inputs rather than diffuse source inputs [27]. This study found *p,p'*-DDT levels up to 80 ng/g in leopard frogs (Ancaster) and 60 ng/g in green frogs (Hillman Marsh); and *p,p'*-DDE levels up to 659 ng/g in leopard frogs (Longpoint) and 754 ng/g in green frogs (Ancaster).

OC residues have been analysed in other amphibian species besides frogs. Mudpuppies (*Necturus maculosus*) collected from the St. Lawrence and Ottawa Rivers in

1988 and 1989 contained a complex mixture of OCs including, DDT, DDE, DDD and dieldrin as well as numerous other compounds [37]. The most prevalent OC was DDE, followed by DDD and then DDT, the concentrations varied between the sites with the St. Lawrence River being higher than the Ottawa River overall [38].

Organophosphate (OP) insecticides represent another chemical class of pesticides and unlike OCs their use is still widespread in North America [28]. There is a dearth of information on OP levels in amphibians, and no studies were found which quantified levels in Ontario wild caught species. Organophosphorus pesticides have generally low persistence in the environment, but they may persist in water and accumulate in certain aquatic vertebrates [21].

Malathion, one of the most commonly used OPs, is a fairly potent insecticide with a relatively low mammalian toxicity as it is rapidly detoxified to nontoxic monoacid derivatives by mammalian carboxyesterases [28]. It is commonly used in the control of mosquitoes, flies, household insects, animal ectoparasites and human lice [39]. Malathion is lipophilic and readily taken up through the skin, respiratory tract, and gastrointestinal tract.

One study which examined malathion uptake in a variety of prey species, suspected that it might be possible for frogs to accumulate OP compounds to high enough levels that predators might be effected [21]. The study reported that tadpoles did accumulate malathion up to 60 times from water, but not to levels that were lethal when consumed in a single meal by ducks [21].

OPs are thought to be preferable to OCs as they appear to breakdown more readily in the environment. However, another study in which detectable levels of

radiolabelled malathion, applied to fields, were found in small rodents, insects and birds, a year after treatment [40].

In summary, there is evidence that OC agricultural contaminants are present in the tissue of Ontario anurans. It is possible that OPs are also present although they have not been analysed for, and are likely to breakdown more quickly.

1.4 Pesticide Effects in Amphibians

Knowledge of pesticide effects on amphibians is largely limited to short term toxicity tests conducted under highly artificial conditions to determine the lethal concentration (LC_{50}) for 50% of the population within a given time. This time period is often very short (1-4 days) [41] and does not consider more subtle effects such as behavioral changes or alterations in immune capacity.

Pesticide effects vary depending upon concentration, method of exposure, and life stage at which the amphibian is exposed [42]. Laboratory studies on both tadpoles and adult frogs indicate that a variety of neuromuscular alterations, developmental abnormalities, altered growth and physiological responses in amphibians occur from exposures to certain OCs. [43-47]

Exposure to organophosphates also causes numerous effects in amphibians. For instance, following exposure of adult *R. pipiens* to concentrations from 50 to 175 ppm of malathion for 15 days, increasing concentrations correlated with mortality, decreased red and white blood cell counts and physical lethargy causing reduced avoidance [48]. The evidence suggests that at high enough doses OC and OP pesticides can cause a variety of effects in amphibians. When more subtle endpoints are examined further, less apparent, though relevant alterations can be seen with low dose exposure. Hormonal and

biochemical endpoints have been used to assess health and measure effects of contaminant exposure in amphibians [16]. There are, however, very few measurements of effects on immune response [39]. It seems likely that immunological alterations will also become apparent when the necessary molecular tools are developed and applied. This thesis uses molecular techniques to measure changes in immune function in pesticide exposed frogs.

1.5 Anuran Immune System

An understanding of the amphibian immune system is necessary in order to determine whether contaminants have a role in altering the systems parameters. The immune system is a structurally and functionally complex system composed of several cell populations and organs strategically placed throughout the hosts body [49]. The structural and functional integrity of the immune system are crucial in performing its protective role against pathogenic agents. Thus any chemically induced alterations may lead to a subsequent change in health of an individual and threaten the status of the population [49].

Amphibian immune defences involve both innate and adaptive components that together lack only a few elements of mammalian immune systems [7]. The innate immune system provides rapid, non-specific protection until the adaptive immune response can be mobilised. As is the case in all vertebrates, amphibians posses phagocytic cells, such as macrophages and neutrophils, that are capable of directly engulfing a pathogen [7, 50].

Specific immune responses include both humoral immunity and cell meditated responses. Organs such as the bone marrow, spleen, thymus, and diffuse lymphoid tissue produce the cellular and humoral components of the immune response [51]. The adaptive immune response requires time to be activated following the detection of a foreign antigen [7]. It is highly specific for a given pathogen and results in the generation of memory and accessory cells [52]. Following the introduction of foreign macromolecules, the production of antigen-specific antibodies by plasma cells derived from B-lymphocytes is referred to as humoral immunity. B-lymphocytes express a unique antigen-binding receptor on their membrane, referred to as a membrane-bound antibody molecule [53]. Antibodies, the major effector molecules of humoral immunity, are glycoproteins consisting of two identical heavy chain polypeptide chains and two identical light polypeptide chains, held together with disulphide bonds. At the amino-terminal end these chains form an antigen binding cleft [53]. These antibodies may be expressed on the B-cell surface as antigen receptors or secreted into the circulation by activated B-cells [54]. Immunoglobulins (Ig) function as antibodies and one type found in amphibians, polymeric IgM is also universally found in all jawed vertebrates [54]. IgM is the first immunoglobulin class produced in the primary response to an antigen.

Cell-mediated immunity is the second arm of the specific immune responses and it involves T-lymphocytes and to a lesser extent macrophages and polymorphonuclear leukocytes. Manifestations of cell-meditated immunity include delayed-type hypersensitivity and resistance to pathogens [Descotes, 1999 #461

1.6 Immunotoxicology

The immune system plays a crucial role in maintaining health. However accumulating evidence indicates that this system can be the target for immunotoxic effects caused by a variety of chemicals [49]. Alterations of the normal immune response usually result in increased susceptibility to viral, bacterial, or parasitic infections and to cancer [49]. The adverse immune system responses on exposure to a xenobiotic substances includes immunosuppression, immunodepression and immunopotentiation [49]. Immunosuppression and immunodepression are detected as marked decreases in humoral, cellular or non-specific aspects of the immune system. Cytostatic drugs, such as cyclophosphimide are known to have immunosuppressive effects and are used as such during organ transplants [55]. Some chemicals can modulate the immune system by increasing the immunological endpoints. Chemically-induced hypersensitivity is defined as an undesirable disproportionate increase in the adaptive immune response following exposure to a chemical [56].

There are numerous adverse immunotoxic effects of xenobiotics including the following:- Organ damage of the immune system can often be observed, such as necrosis; Multiple histological effects in the thymus, the bone marrow, and the lymph nodes; Chemically- induced cellular pathology, including abnormal proliferation of stem cells in the bone marrow; altered maturation of immunocompetent cells and changes in B and T cell subpopulations; Functional alterations of immunocompetent cells generally classified as altered humoral immunity, cell-mediated immunity, or non-specific responses; A two directional interaction of the immune system with xenobiotic

detoxification and biotransformation mechanisms, generally observed as an impairment of chemical elimination in immunodeficient individuals [49].

In this thesis some of these parameters were assayed in pesticide exposed frogs and others are suggested for future study.

1.7 Assays Of Immunological Function

Assays of immunological function can be divided into *in vivo* and *in vitro* responses. The studies presented in this thesis use an *in vivo* approach to assay immunological function in pesticide exposed frogs. *In vivo* models have the advantage of integrating the immune response at the level of the whole animal, and therefore take into account indirect influences on the immune response, such as those derived from neurological or endocrine adverse effects of xenobiotics [57].

A good correlate of cell-meditated immunity is the delayed-type hypersensitivity (DTH) reaction. This assay was used in the studies presented in this thesis (the methodology is presented chapter 2, section 2.2.6, page 27). The assay requires the specific recognition of a given antigen by activated T-lymphocytes, which subsequently proliferate and release cytokines. These cytokines increase vascular permeability, induce vasodilatation and macrophage accumulation, and finally antigen destruction [57].

Examination of the innate immune system can be achieved by investigating phagocytic pathways. The role of phagocytosis is primarily the removal of microorganisms and foreign bodies, but also the elimination of dead and injured cells [57]. Five major successive steps of phagocytosis can be identified, these are chemotaxis, adhesion, ingestion, killing of microbial pathogens and metabolic activation

of phagocytes. Ingestion (appendix B, page 120, secion A5.6.2) and the killing of microbial pathogens (method, chapeter 2, section 2.2.7, page 27) were examined in pesticide exposed frogs. The killing of microbial pathogens is the result of the oxidative burst in phagocytes [57] and can be examined using a chemiluminescent technique. Phagocyte activation results in the release of variety of reactive oxygen compounds by the NADPD-oxidase complex and myeloperoxide [58]. These processes generate electronically excited states, which on relaxation to the ground state emit photons. This can be measured from a whole blood sample using a luminometer provided that the reaction is amplified by the addition of luminol and by using zymosan to induce the oxygen radical production. This method is a reliable tool for studying the metabolic responses of phagocytes previously exposed to chemicals [57]. The whole blood chemiluminescence in *Rana sp.* mainly reflects neutrophil activity, since they are the most abundant blood phagocytes, but eosinophils and monocytes are also represented [58].

Humoral immunitiy can be assessed by the measurement of antigen specific antibody titers in serum. Antibody responses require B cells to mature into plasma cells to synthesise and release antibodies. Most, but not all antibody responses are under the control of T lymphocytes [57]. A variety of T-dependant antigens such as keyhole limpet haemocyanin (KLH), bovine serum albumin (BSA) and ovalbumin are commonly used to quantify the production of serum antibodies. KLH is a strong protein antigen which can induce either humoral or cellular immune responses [57]. This was assessed in the pesticide exposed frogs in this thesis using the enzyme-linked immunosorbent assay (ELISA) and the methodology is presented in section 2.2.5, page 26. The ELISA uses an

enzymatic reaction to detect the specific binding of an antibody with an antigen. This is an easy and relatively quick assay [57, 59] that can be performed using small volumes of serum on a 96 well plate. When an appropriate monoclonal antibody is available, the ELISA is highly sensitive and specific compared to immunoprecipitation methods, agglutination methods and light scattering techniques [59].

1.8 Laboratory Studies Examining the Effects of Pesticides on Immune Function

There is extensive literature on immunotoxicity studies that have been conducted under laboratory conditions in rodents [60]. Much less has been undertaken on other organisms and results on rodents are not always indicative of what will happen over a broad species range as the effects of pesticides on the immune system of different animals may vary widely. Nonetheless the existing literature provides a introductory point with which to base further studies.

For example, DDT was one of the first pesticides studied for its effects on the immune system [28] and has shown contradictory results. Some studies indicate that immune response is not an important target of this class of insecticide [61] while others show marked changes in humoral endpoints. In rabbits [62] mice [63] and chickens [62] there was no effect on antibody titer to an oral dose of DDT. Paradoxically Banerjee *el al.* [64] report that mice fed a diet spiked with up to 100 ppm DDT daily for 3 to 12 weeks show a decrease in primary anti-sheep red blood cells (SRBCs) IgM and IgG serum antibody levels. A decrease was also seen in serum anti-SRBC IgG with a concurrent increase in serum anti-SRBC IgM in chickens fed a DDT spiked diet (100 ppm) *ad libitum* from hatching to 40 days [65].

Similarly, dieldrin has been used in several immunotoxicological studies on numerous species. It has been found to reduce the number of antibody-producing cells and antibody production in mice [66, 67]. Macrophages have been implicated as the primary target for dieldrin immunotoxicity in mice [28]. The antibody responses of inbred mice to SRBCs, *Salmonella typhimurium*, and lipopolysaccharide (LPS) were all depressed at a dieldrin dose of 0.6 LD_{50} [66]. Dieldrin also appears to decrease the ability of macrophages to phagocytose and kill *S. typhimurium*, [68]. Peritoneal macrophages from dieldrin exposed mice showed effects on antigen processing and presentation [69, 70] as they had decreased ability to uptake avidin or to release the processed antigen.

In general, there is relatively little information available on the effects of organophosphorous compounds on the immune system and the majority of the studies have been conducted on laboratory animals [28]. In many cases malathion was found to cause suppression of the immune system. For example, administration of several low doses of malathion in mice for prolonged periods of time, which results in an overall high-dose exposure, was shown to decrease the level of humoral responses [28]. Similarly, exposure of 10 mg/kg per day of malathion in rabbits for 5-6 weeks resulted in significantly decreased antibody titers generated in response to *Salmonella typhi* vaccination [71]. In contrast a single dose of malathion administrated to mice, 2 days after immunization with SRBCs suppressed a primary IgM response, but not the IgG and the study suggested that that cholinergic poisoning may have resulted in a stress-related suppression of the immune system [72]. At higher concentrations, malathion was also found to inhibit the respiratory burst activity and the proliferative response to mitogens

of murine splenocytes after *in vitro* exposure [73, 74]. In addition, the respiratory burst in response to phorbol of murine peritoneal cells was altered by malathion exposure [73]. It is important to note that technical grade malathion is contaminated with O,O,S,-trimethyl phosphorothioate (OOS-TMP) [61]. OOS-TMP has been found to block antibody response, in mice, although the effects were reversible [75]. It is possible that some reports of humoral and cell mediated suppression which are reported to be attributed to malathion may in fact be OOS-TMP mediated suppression.

In other cases, enhancement of the immune function is detected after exposure to malathion. Malathion has been reported to stimulate macrophage activity and the primary humoral-mediated response in mammals [61]. For example, after oral administration of noncholinergic doses of malathion an increase in humoral responsiveness was detected after 5 days and proliferative response to mitogens was enhanced on all days following acute exposure [76]. Malathion appears to effect the function of the adherent cell population, at the lowest observed acute effect level (LOAEL) of 0.25 mg/kg, respiratory burst of peritoneal leukocytes were elevated [73]. This is possibly through the degranulation of peritoneal mast cells and the subsequent exposure of peritoneal cells to mast cell mediators [28]. In summary, malathion suppresses the generation of an immune response at high doses, possibly through the effects of the stress of such a toxic exposure. At lower doses however, malathion appears to elevate the immune response [28].

1.9 Link Between Disease Outbreaks and Immunosuppression

There are numerous diseases that can afflict amphibians and some have been hypothesised to be a contributing factor in some declines [1, 7, 12]. The bacteria, *Aeromonas hydrophila* has been associated with affliction in frogs [77, 78], the disease has been termed "red-leg disease" [78], although the signs are not sufficiently consistent or specific to justify the term [77]. Red-leg describes symptoms but a definitive causative agent is missing and might vary from case to case. There are concerns that immunosuppression due to contamination could be a cause for an increased predisposition to infection [79, 80]. It has been postulated that mortality and the disappearance of certain amphibian populations have been due to bacterial disease subsequent to reduction in immune function [81]. Several incidents of mass mortality have been attributed to bacteria such as *Aeromonas hydrophilia* and various fungus species [1, 19, 82, 83]. The presence of an underlying factor rendering the frogs susceptible to disease is likely as bacteria such as *A. hydrophila* are ubiquitous in the amphibian environment [84] and can be cultured from healthy animals [85, 86]. It is known that organochlorine insecticides can also significantly enhance response to immune system stimuli [87].

1.10 The Effect of Stress on the Immune System

Although stress hormones were not examined in the studies presented in this thesis, it has important implications to immune capability and should be addressed. Stress can alter immune function and make animals more susceptible to infection. The

immune system is intertwined with the endocrine and nervous system with dynamic interactions between genetic information and various organ systems some of which are intimately involved in the effects of environmental agents [88]. The inter-relationship between the immune system and adrenal function has been demonstrated in rats which were exposed to acute noise alterations they were found to have increased plasma corticosterone levels and showed definite alterations in immune functions [89].

In mammals and fish even short term stress can induce immunosuppression lasting several days [84]. This is mediated through increases in levels of endogenous corticosteroids such as corticosterone, leading to lymphoid cells depletion, and attenuation of the response to antigenic challenge [90]. There is increasing evidence of physiological links between neural pathways, the endocrine system and the immune system apart from the known immunosuppressive effects of glucocorticoids. The effects of environmental contaminants on the endocrine system have recently become apparent and it is possible that the link between toxins and the immune system could be elaborated through other organ systems such as the brain, the autonomic nervous pathways and the endocrine system [84].

Corticosteriods have been found to have a role in regulating fluctuations in the immune function of ectothermic vertebrates [91]. This role was supported by the correlation between levels of circulating coticosteroids and the strength of immune responses. Corticosteroids are known to exert a plethora of additional effects on ectotherm immune activities. They can have both an anti-inflammatory or inflammatory immune responses depending upon the concentration, physical state of the animal, timing of exposure and the immune assay being tested [91].

Studies, which have examined the effect of stress on amphibians, have found that increased corticosteroids can alter the immune function of this group as well [54]. Stress can be caused by the toxic burden of a contaminant loading and this can alter the immune function even if the compound itself is not mechanistically responsible. Stimulated immune activity can be induced by suppression of corticosterone, which is sensitive to contaminant exposure [87]. Increased disease susceptibility follow contaminant exposure was examined in Woodhouse's toads (*Bufo woodhousi*) [39]. It was found that clinical disease, hepatomegaly, and death occurred at a higher rate when toads were exposed to malathion and then challenged with *A. hyprophila* compared to those that were exposed to the malathion but not challenged with the bacteria.

1.11 IMMUNOSUPPRESSION IN MICROCOSM STUDIES AND WILDLIFE

Numerous studies have examined a variety of immunological parameters in both field and microcosm studies. For example harbour seals fed a diet of contaminated herring from the Baltic exhibited immunosuppression compared to a control group fed cleaner Atlantic herring [92]. Wildlife studies have established strong linkages between contaminants and alterations in immune function in caspian terns, herring gulls [93], bottlenosed dolphins [94] and beluga whales [95].

Exposure response relationships have also been demonstrated in a number of species, for example trout exposed in a microcosm to varying concentrations of creosote resulted in both stimulatory and suppressive effects in immune function [96].For instance, when the immunotoxicity of creosote to rainbow trout (*Oncorhynchus mykiss*) was evaluated in a microcosm both stimulatory and suppressive effects were observed.

Following 28 days of exposure to up to 100 µl/L of creosote, reductions in leukocyte oxidative burst, plasma lysozyme levels. blastogenesis in response to LPS and the number of circulating immunoglobuiins were observed. Phagocytic activity was enhanced marginally in exposed fish. Blastogenesis in response to phytohemagglutinin (PHA) and concanavlin A (Con A) were unaffected. The authors concluded that environmental concentrations of polycyclic aromatic hydrocarbons (PAHs) can impair fish immune parameters [97]. Another microcosm study investigated the effect of untreated chlorine free bleached pulp effluents on the immune defense of the roach (*Rutilus rutilus*). After 5 weeks of exposure in up to 20% effluents immunosuppression was detected. Formulation of specific antibodies, and antibody secreting cells were suppressed in the spleen and plasma of highly exposed fish. Similarly Con A stimulated proliferative responses, respiratory burst and leukocyte migration were decreased. The results demonstrated that untreated pulp effluents significantly affected the immune parameters of the roach [98].

A longer term captive feeding experiment examining the effect of contaminants on the immune function of harbour seals (*Phoca vitulina*). It was found that the seals consuming herring from the Baltic Sea (contaminated) had altered immune responses than the group feed Atlantic (uncontaminated) herring. By way of illustration, they exhibited diminished natural killer cell function, lower proliferative responses of peripheral blood to Con A, PHA and pokeweed mitogen (PWM), impaired DTH to ovalbumin and had elevated circulating neutrophils [99].

Some studies have examined the effects of contaminant association with immune functions. For example, contaminant-associated immunosuppression was examined in

prefledgling herring gulls (*Larus argentatus*) and Caspian terns (*Sterna caspia*) from the Great Lakes. Evidence of suppression T-cell mediated immunity as determined by a PHA skin scratch was correlated organochlorine levels in eggs. Biologically significant differences in anti- SRBCs antibody titers were detected, although these were not correlated to organochlorine levels. This study concluded that the suppression of T-cell mediated immune responses were associated with exposure to organochlorine contaminants [93]. In another study the influence of xenobiotics on immune parameters of spot (*Leiostomus xanthurus*) in Chesapeake Bay was determined in fish collected from five sites that represented a gradient of sediment PAH concentrations. Proliferative responses to the T-cell mitogens Con A and PHA were suppressed whereas the B-cell mitogen LPS, PWM and peanut agglutination were elevated [100]. A study in the Mediterranean which examined free-ranging dolphins (*Tursiops truncatus*) in 1991, found them to have reduced peripheral blood lymphocyte responses to Con A and PHA which were correlated to increasing whole blood concentrations of several contaminants, including DDT and DDE [94]. Another study, which focused on wild birds, looked at species which were exposed to pesticides and found stimulatory rather than suppressive effects. Tree swallow chicks reared in pesticide sprayed orchards showed significantly stimulated T- and B-cell blastogenic response and a reduction in thymic maturation which correlated with increasing pesticides. In this study pesticides, polychlorinated biphenyls, lead and arsenic were measured in eggs and were all found to be low except for *p,p'*-DDE, which was as high as 2.29 μg/g wet weight. The frequency of the pesticide exposure in these birds was correlated with the immunostimulatory effects found [87].

1.12 THESIS RATIONALE

The studies outlined confirm that environmental xenobiotics have the potential to alter the immune function of a variety of species and specifically, that there are few studies which address alterations of the immune function of amphibians, especially at post metamorphic stages. Within Ontario the regions which have recently experienced declines tend to be in agricultural areas rather than industrial or urban sites. In addition, contaminant analysis had shown that pesticides residues in Ontario frogs are higher from agricultural sites [27]. Therefore, pesticides were examined initially rather than industrial contaminants, as leopard frogs predominately inhabit agricultural areas and are exposed during all life stages.

CHAPTER 2.0 - PESTICIDE-INDUCED IMMUNE SUPPRESSION OF THE NORTHERN LEOPARD FROG (*Rana pipiens*)

2.1 INTRODUCTION

Amphibian populations are in decline worldwide [12, 101, 102], and in a number of these cases, infectious disease has been demonstrated to be the proximal cause of death [7]. Local declines and extirpations of amphibian populations have been associated with bacterial infections which are ubiquitous in freshwater and immunosuppressed individuals are reportedly more susceptible [103]. Though many hypotheses have been proposed to account for population declines and disappearances, in most cases the cause or causes are still uncertain [12, 42]. As a result of their thin, permeable skins and prolonged exposure, first in their aquatic and then in their terrestrial life stages, amphibians are thought to be susceptible to contaminants in their environment [18]. Organochlorine pesticide use in North America has decreased since the 1970s, but pesticides still pose a threat to biota because of their toxicity, environmental persistence, and potential to bioaccumulate in foodchains [36]. The disappearance of the threatened California red-legged frog was found to have strong positive association with percentage upwind agricultural land use [104]. The historic application of DDT to wetlands for mosquito control may have been important in affecting amphibian populations [105]. Since 1972 three species of amphibians have become locally extinct in Essex County, and a fourth species has been reduced in abundance in Point Pelee National Park [36]. DDT and dieldrin have recently been found in the tissues of frogs from Point Pelee [27, 36] at concentrations up to 160 μg/kg and 199 μg/kg respectively.

Many chemicals introduced into the environment by human activity have the capacity to disturb the immune system of wildlife and humans. Experimental studies demonstrate that exposure to synthetic chemicals can result in increases or decreases in measured immune parameters and hypersensitivity [106]. A number of organochlorine pesticides, such as DDT and dieldrin, and organophosphate pesticides such as malathion, have the potential to produce immunotoxicity in a variety of species [106]. Experimental results are consistent with wildlife studies that have demonstrated contaminant-induced immunosuppression. There are numerous studies which quantify the levels of different compounds in amphibians, but relatively few which address the sublethal effects. One study on Woodhouse toads exposed to a dermal dose of malathion and then injected with *Aeromonas hydrophila* had clinical disease, hepatomegaly and increased mortality than control toads [39].

This study investigated whether existing mammalian and avian immunological assays, could be adapted to quantify alterations in immune function of amphibians caused by pesticides. The immunological methods developed were non-lethal. This allows for repetitive sampling of animals within a population, the feasibility of a long term monitoring program, and the quantification of subtle sub lethal effects, which has not been conducted on amphibians to date [16].

Following the development and optimisation of relevant amphibian immune assays, an injection study, a dose response study and a reverse order immunisation study were initiated. The aim of the injection study was to determine whether a sublethal dose of a known immunosuppressant pesticide would elicit immune alterations in adult frogs. The dose response study used DDT to determine at what level the alterations could be

measured. The reverse order immunisation study determined whether individuals could mount an immune response if they were immunized prior to pesticide exposure. To date there have been few studies examining immune function in a non-sacrificial manner to assess the health of pesticide exposed amphibian populations.

2.2 MATERIALS AND METHODS

2.2.1 Frog Husbandry

Northern leopard frogs (*Rana pipiens*) were captured from the Collingwood area of Ontario in 1999 and maintained in the Aquatic Facility at the Great Lakes Institute for Environmental Research at the University of Windsor for one year prior to use. The frogs were housed in aquaria (30 x 90 cm) in groups of five and provided with a washed concrete block covered in rubber matting as a feeding platform. Underwater charcoal filters (Quick~Filter® (802), Hagen) were utilised to remove waste products from the water. The tanks were emptied and scrubbed once a week and refilled with chlorinated tap water. Frogs were fed daily with crickets that were maintained on a diet of bird granules (Tropican, Hagen, Inc, Montreal). Individuals were identified using a combination of close up photographs, line drawings of markings accompanied by a detailed written description and leg tattoos using acrylic paint.

2.2.2 Experimental Design

Changes in immune function were induced in the frogs by subcutaneous injection of DDT, dieldrin, malathion and cyclophosphamide (Cy). It was not necessary to undertake range finding for the following sublethal doses have been published:- DDT

and dieldrin doses for *R. pipiens* have been reviewed by Harfenist *et al.*, [46]. Sublethal malathion doses were taken from the study dealing with Woodhouses toads [39] and Cy was determined for *R. pipiens* from the levels used by Bugbee *et al.*, [107], although for this compound, a lower dose was used than the earlier study.

For the injection study, stock solutions of 250 mg/L *o'p*, DDT, (Aldrich, Chemical Co., Milwaukee, WI, USA), 25 mg/L dieldrin (Sigma-Aldrich Chemical Company, St. Louis, MO, USA), 330 mg/L of malathion (White Rose, Windsor, ON, Canada), and 3000 mg/L Cy (Sigma-Aldrich Chemical Company, St. Louis, MO, USA), were made up in dimethyl sulphoxide (DMSO). Each group of frogs (n=5) was exposed to an individual compound by an intramuscular injection in the right thigh with 3 μl/g bodyweight of each pesticide solution. The treatment groups were as follows; group 1 *o'p*,DDT; group 2 malathion; group 3 dieldrin; group 4 Cy as the positive control and group 5 DMSO, the carrier for all administrations as the negative control.

A dose response study was initiated using *o'p*,DDT. Stock solutions of 0, 0.25, 2.5, 5, 10 and 25 mg/L DDT in DMSO were made up. The treatment groups received: group 1, 25 mg/L DDT stock; group 2, 2.5 mg/L DDT stock; group 3, 0.25 mg/L DDT stock and group 4, 0.025 mg/L DDT stock solution. Each group of frogs was exposed to the appropriate solution by an intramuscular injection in the right thigh with 3 μl/g bodyweight of each pesticide solution.

A reverse immunisation order experiment was also undertaken, using three exposure groups containing four frogs per group. Stock solutions used were 25 mg/L DDTin DMSO, 300 mg/L Cy in DMSO and DMSO only control group. Each group of

frogs was exposed to the appropriate solution by an intramuscular injection in the right thigh with 3 µl/g bodyweight of each pesticide solution.

2.2.3 Immunization

Two days after exposure to the relevant xenobiotic, a stock solution containing 2 mg/mL of keyhole limpet hemocyanin linked to dinitrophenyl (KLH-DNP) (Calbiochem, La Jolla, CA); 2mg/ phytohemaggutinin (PHA-P), (Sigma-Aldrich Chemical Company, St. Louis, MO, USA) and 16 mg/mL of dimethyldioctadecylammonium bromide (DDA), (Fluka chemika, Ronkonkoma, NY,), was prepared. Titermax gold (Sigma-Aldrich Chemical Co., St. Louis, MO, USA) was used as the adjuvant at a ratio of 2.5 mL:1 solution:adjuvant. Each frog received 100 µl of this emulsion intramuscularly in the left thigh.

To determine the importance of the immunisation order, a study was initiated using three groups of frogs. These were immunised with the mixture outlined above then, five days later, they were injected with: group 1, 25 mg/L DDT stock solution; group 2, 300 mg/L Cy; group 3, DMSO control. Five days after immunisation the delayed type hypersensitivity reaction (DTH), (see section 2.2.6) was initiated and three days after that the frogs were bled and the enzyme linked immunosorbent assay (ELISA) (section 2.2.5) and chemiluminescence assay (CL) (section 2.2.7) performed.

2.2.4 Blood Sampling

Blood was collected by cardiac puncture using a 28G 5/8 and a 1cc tuberculin syringe. The anti-coagulant used was lithium heparin (10 000 units/mL deionized water),

(Sigma-Aldrich Chemical Company, St. Louis, MO, USA). During the procedure the frogs were immobilised by wrapping in damp paper towels with an opening over the sternum. After collection blood was stored in a 1.5 mL microcentrifuge tube at 4°C until used.

2.2.5 Antibody Response to KLH-DNP

Microtiter plates (96 well) were pre- coated overnight with KLH in coating buffer (15mM Na_2CO_3, 34.88mM $NaHCO_3$, 3.125mM NaN_3 pH 9.6) and incubated at room temperature. The KLH solution was retained, stored at 4°C and reused ten times. The plate was washed three times using Tris buffered saline solution (TBS-T) (136.89mM NaCl, 2.68 mM KCl, 24.76mM Tris base, 0.5 ml/LTween, pH 8) in a squeeze bottle. 300 µl blocking buffer (5% gelatin in TBS-T) was added to all wells and the plate was incubated for 1 hour at 37°C. The plate was washed three times using TBS-T. The blood samples were centrifuged (2000 rpm for 5 min at 4°C) in 1 mL eppendorf tubes and the plasma removed. A 100 µl aliquot of plasma was placed into appropriate wells in replicates of four. The plate was washed three times with TBS-T. The primary antibody used was 6-16 [108], a mouse monoclonal antibody that was raised against *Xenopus* anti IgM. 100 µl of antibody solution was added undiluted to all wells and incubated at 37°C for 1 ½ hours. The plate was again washed three times with TBT-T. Next 100 µl of secondary antibody solution (1:1000 in TBS-T) was added and the plate incubated for 1 hour at 37°C. The secondary antibody was anti-mouse IgG (whole molecule) alkaline phosphatase conjugate, developed in goat, (Sigma Chemical Company, St. Louis, MO, USA). The plate was washed three times with TBS-T and 50 µl of alkaline phosphatase

developing solution (Sigma Fast p-nitrophenyl phosphate tablet set, Sigma Chemical Company, St. Louis, MO, USA) added to all wells. After 30 minutes 50µl of 0.03 M NaOH was added as stop solution. Three control wells were set up in replicates of four on each plate, one that contained fetal calf serum (Gibco, BRL) in place of frog plasma, the other two wells contained primary and secondary antibody only. Results were read on a plate reader (Biolinx, V2.20, Dynatech) at 405 nanometers (nm).

2.2.6 Delayed Type Hypersensitivity Skin Response to PHA-P

Nine days after immunisation a solution of PHA-P, 2mg/mL in phosphate buffered saline (PBS) was prepared. The thickness of the middle toe of the right and left hind foot at the point where the webbing ends was measured using an electronic micrometer (Digimatic Outside Micrometer 0-1", Mitutoyo, Morgan Precision Tools, Aurora, IL, USA). The right toe was injected (1/2 cc Micro-Fine insulin syringe, 0.33 x 13mm/ 29G x ½, Becton Dickinson, NJ, USA) with the PHA-P solution and the left toe was injected with the PBS to determine the non-specific inflammation. Measurements of both sites were taken 24, 48 and 72 hours after injection. The area to be measured was extremely small and the sensitivity and accuracy of this method was determined in a double blind measurement study using, unexposed frogs in a four-day trial.

2.2.7 Chemiluminescence

The zymosan induced chemiluminescence of whole blood was determined using the method developed by Marnila *et al.*, [58]. The assay was performed on a 96 well microtiter plate in replicates of four withi n ten hours of the blood sample being taken. Each well contained whole blood (500 nl) diluted in 200 µl of frog Ringer's solution (116

mM NaCl, 1.2 mM KCl, 1 mM $CaCl_2$, 2.70 mM $NaHCO_3$), and 1 mM Luminol (5-amino-2,3-dihydro1,4-phthalazinedione, Sigma-Aldrich Chemical Company, St. Louis, MO, USA). The contents of the four wells were made up in an eppendorf, vortexed lightly and a 225 µl aliquot added to each well. Directly prior to reading the plate 500 µg of Zymosan (Zymosan A, Sigma-Aldrich Chemical Company, St. Louis, MO, USA) was added. Each well had a total volume of 250 µl. The chemiluminescent emissions were measured at 3 minute intervals for 30 readings to obtain kinetic curves on a luminometer (MLX Microtiter Plate Luminometer, Dynex Technologies, Chantilly, VA). Results were represented as relative right units (rlu). The peak chemiluminescence was used as the sample value.

2.2.8 Contaminant Analysis

Frogs were sacrificed at three time points throughout the injection study, 2 days, 8 weeks and 20 weeks after exposure to the relevant compound for the group. Sample preparation was done using an adaptation of the method described by Lazar [109]. Briefly the whole frogs were homogenised and stored in chemically clean glassware. Carcass homogenates (1 to 2 g) were ground with 20 g Na_2SO_4. The homogenate was wet packed in a 2.1 cm i.d x 24 glass column containing 50 mL dichloromethane;hexane (1:1) and a surrogate spike of 500 µl tri-methyl-benzene, and for the DDT groups, 100 µl methyl-sulphone was added. After 1 hour, the solvent was eluted from the column and the homogenates extracted with a further 250 mL DCM:hexane (1:1). The extracts were evaporated to 2 mL, a 10% aliquot was removed for lipid determination by gravimetric analysis, and the remaining extracts cleaned up by gel permeation chromatography [110].

Clean up of concentrated extracts were performed by florisil chromatography. Concentrated extracts were loaded onto 1 cm i.d. x 24 glass columns wet packed with 6 g activated florisil (BDH Inc, Toronto, ON, Canada) and a 1 cm Na_2SO_4 cap. The relevant compounds were eluted from the florosil with 50 mL hexane, followed by 50 mL of 15%DCM/hexane, which was followed by 130 mL 60% DCM/heaxane. The fourth and final fraction was eluted with 80 mL 7:93 methanol:DCM as the fourth to examine methyl-sulfones.

Gas chromatographic analysis was performed on a Hewlett-Packard 5890 GC equipped with a 63 Ni electron capture detector and HP-7673 autosampler. The column was a 30-m DB-5 fused silica capillary column (0.25-μm film thickness, J&W Scientific). Organochlorines were identified by retention time and quantification of individual compounds was based on the response factors from a well characterised standard mixture [111]. Standards were injected for every 5 samples analysed and duplicates were added after the sixth sample.

2.2.9 Statistical Treatment

All statistics were performed using Systat version 7.0. Differences between the groups were determined using analysis of variance (ANOVA), a Bonferroni multiple comparison test based on Student's t statistic was used to establish which of those groups were distinct from each other by adjusting the observed significance level for the fact that multiple comparisons are made. Outliers were omitted according to Studentized residual values generated by Systat.

2.3 RESULTS

2.3.1 Antibody Response to KLH-DNP

The antibody response to KLH for the pesticide injection study is presented in Figure 2.1. The amount of KLH specific IgM produced by the frogs was significantly lower ($p = <0.0001$), for the both pesticide and positive control groups at 2, 4 and 8 weeks after exposure compared to the DMSO control frogs. Recovery of IgM levels for all of the groups occurred between 8 and 20 weeks after initial exposure. To account for variation in the assay reaction at different time periods the results are expressed as a percentage of the control frogs (DMSO exposed). Two weeks after exposure the groups injected with DDT, malathion and Cy produced negligible quantities of the antibody (1-2 % of DMSO). The dieldrin exposed frogs produced about 30% of the levels of antibody produced by the DMSO control frogs. By week four the mean KLH specific antibody levels were slightly higher for the DDT, malathion and Cy exposed groups (4-11%) but little change in the dieldrin exposed frogs, which remained around 30% of the DMSO control frogs. Further recovery of the with DDT and dieldrin exposed animals was evident by week eight (30 and 38% of DMSO respectively) although the malathion exposed group remained low (< 4% of DMSO). By week twenty recovery of IgM levels had occurred in all pesticide exposed groups as measured by antibody production. The

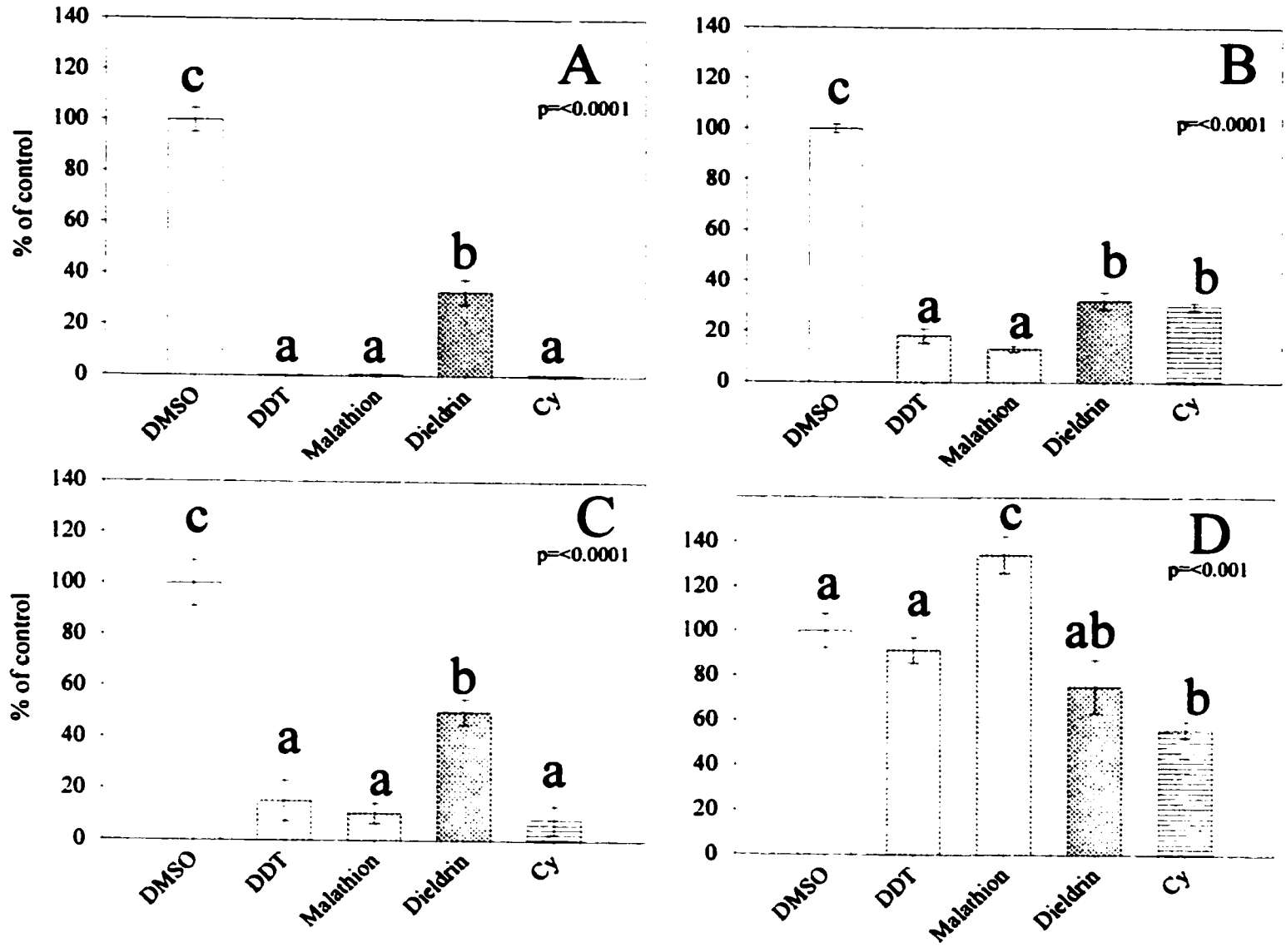

Figure 2.1 – Anti KLH IgM levels as determined by ELISA, in serum of leopard frogs A) 2 weeks, B) 4 weeks, C) 8 weeks and D) 20 weeks after exposure to DMSO, DDT, dieldrin, malathion and Cy. n=5 per group

IgM levels of the Cy exposed group, however remained depressed at 56% of the DMSO control frogs. Malathion (130%) reacted to a greater extent than did the DMSO group, and the values for DDT and dieldrin (91% - 74%) were much higher than previous weeks.

A comparable antibody production trend was apparent during the dose response study, although it was not dose dependant at the concentrations administered. The results of the KLH specific IgM production in the dose response study are shown in Figure 2.2. Two weeks after exposure to DDT the higher dose groups (25 and 2.5 mg/L DDT stock), and the lower dose groups (0.25 and 0.025 mg/L DDT stock) had anti-KLH IgM antibody titers which were significantly ($p<0.0001$) lower than the DMSO control group. Four weeks after exposure there was little statistical difference between the antibody titers of the groups ($p=0.023$), although the lowest exposure group (0.025 mg/L DDT stock) was slightly depressed in comparison to the other groups. Eight weeks and

ten weeks after exposure the anti-KLH IgM levels remained similar ($p=0.411$ and 0.188 respectively) for all groups.

When frogs were administered KLH-DNP five days prior to exposure to DDT antibody suppression was no longer apparent. Figure 2.3 shows the anti-KLH IgM ELISA results when the immunisation order was reversed. All exposure groups were statistically similar ($p=0.213$) at both 2 and 4 four weeks after exposure.

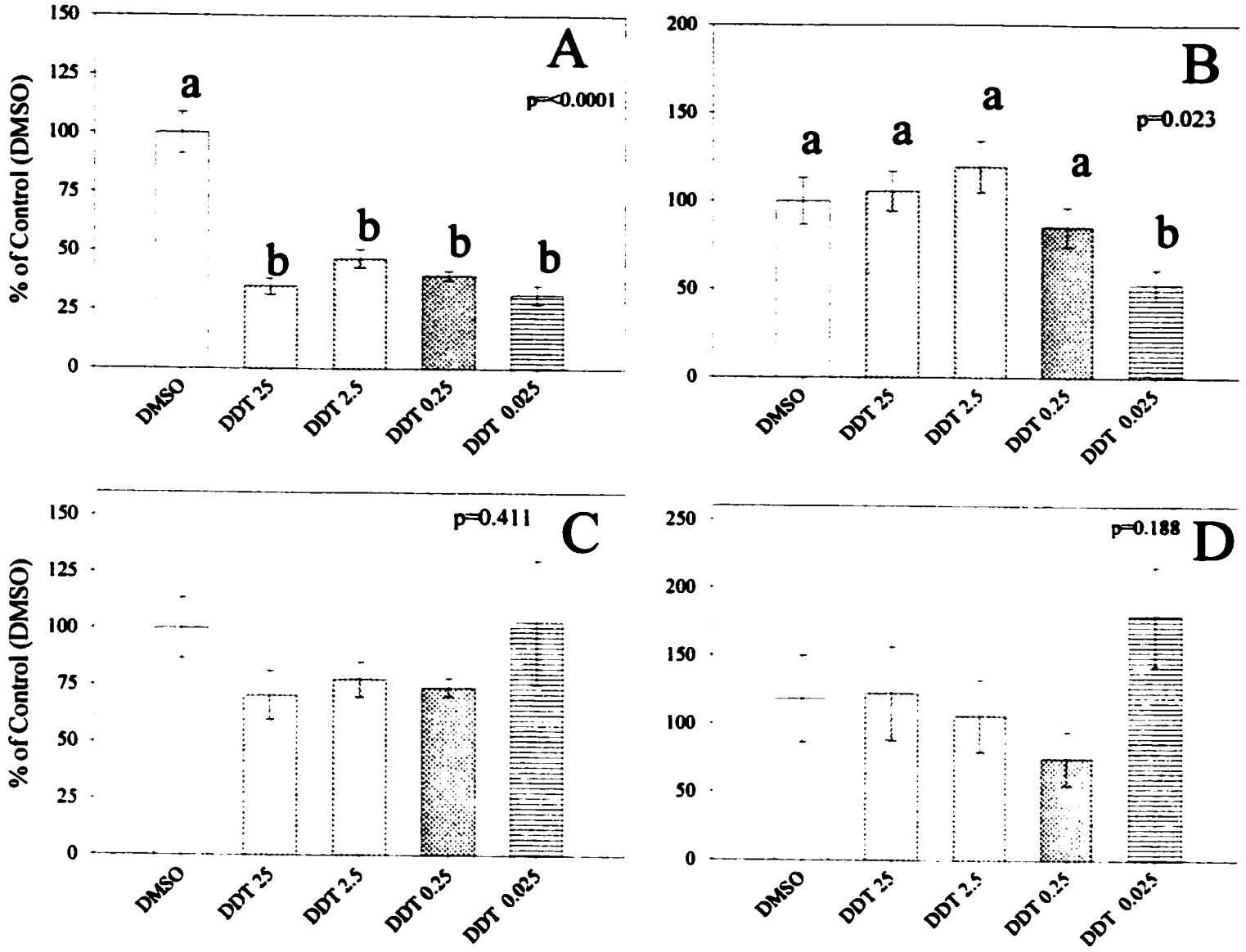

Figure 2.2 - Anti KLH IgM levels as determined by ELISA, in serum of leopard frogs A) 2 weeks, B) 4 weeks, C) 8 weeks and D) 12 weeks after exposure to stock solutions of 25, 2.5, 0.25, 0.025 and 0 mg/L of DDT in DMSO.

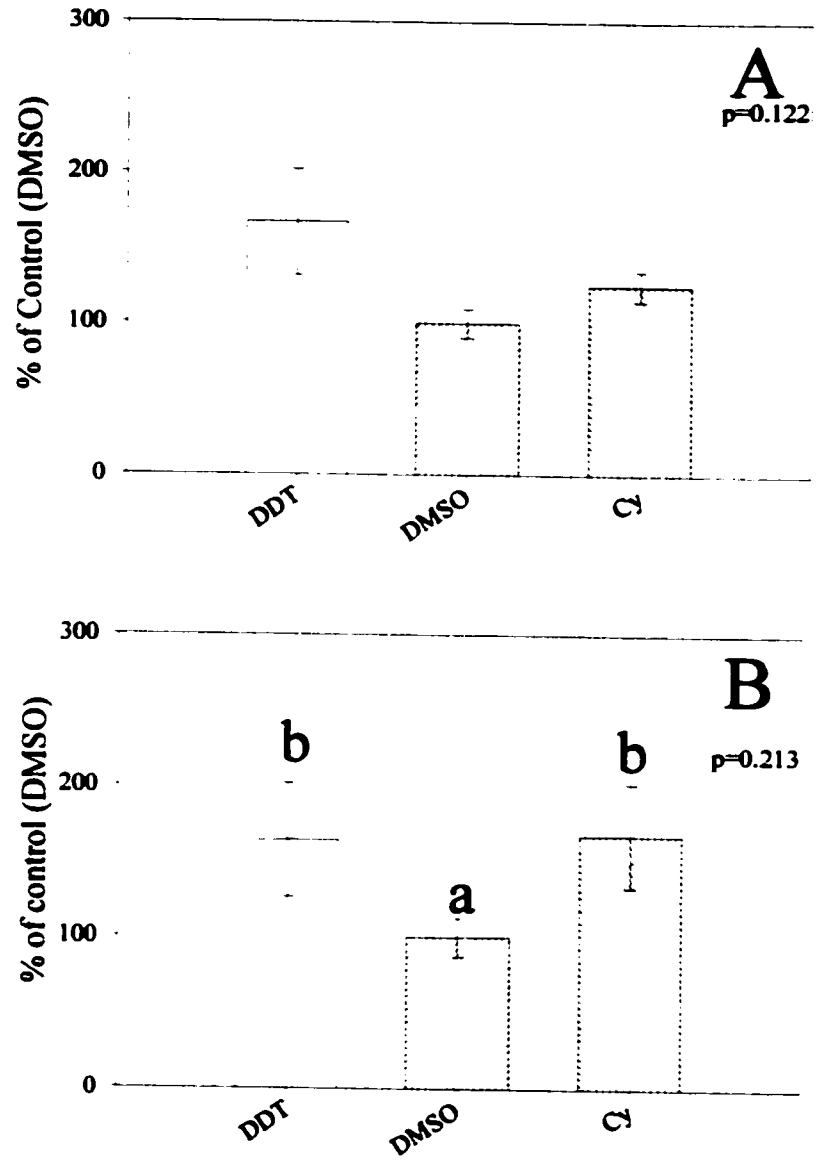

Figure 2.3 – Anti-KLH IgM levels as determined by ELISA A) 2weeks and B) 4 weeks after contaminant exposure in the reverse order immunization study

2.3.2 Delayed-Type Hypersensitivity to PHA-P

The DTH results from the injection study, as shown by the mean increase in toe thickness (24 hour measurement – initial measurement) is illustrated in Figure 2.4. The pattern, indicates that the pesticide exposed frogs had a higher response than the DMSO control frogs, although the results are not significant ($p=0.597$). The DTH reaction was initiated two weeks after contaminant exposure. The effect of repeated sampling on a single toe was unknown so this assay was undertaken only once. The DTH optimisation study found that with repeated sampling in a blind study the standard deviation from the mean was 0.165 ± 0.09. The results are plotted as the difference in thickness (initial-24 hour measurement) of the right (PHA injected) toe minus the left (PBS injected) toe at 24 hours.

The DTH from the dose response study is shown in Figure 2.5 and shows a similar pattern to the injection study. Similarly the standard error is too great to show statistical significance ($p=0.743$), but the group which was exposed to the highest concentration of DDT (25 mg/L DDT stock solution), shows the greatest increase in toe thickness. This continues in a dose dependant manner, until the lowest exposure group (0.025 mg/L DDT stock solution) has a response that is similar to the DMSO group.

Figure 2.6 shows the response to PHA-P after the initiation of the DTH assay when the order of immunisation precedes exposure by five days. Exposure groups of DDT (25 mg/L stock solution), DMSO (control) and Cy (positive control) produced a reaction which is opposite to the pattern seen in the dose response and injection studies although no statistical differences exist ($p= 0.743$). The DMSO produced the highest reaction with lower reactions produced by the DDT and Cy groups.

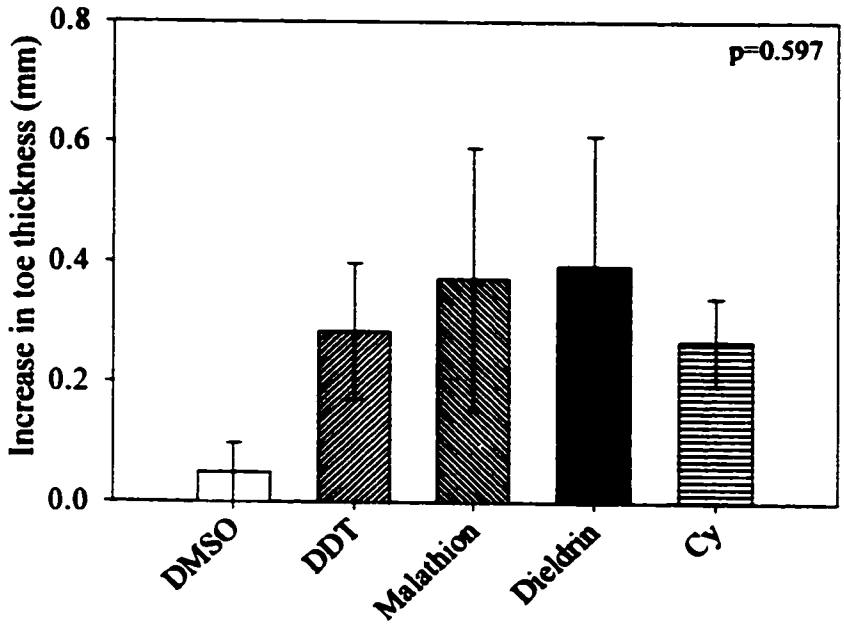

Figure 2.4 – Delayed type hypersensitivity as determined by change in toe thickness in response to PHA, in leopard frogs 2 weeks after exposure to DMSO, DDT, dieldrin, malathion and Cy.

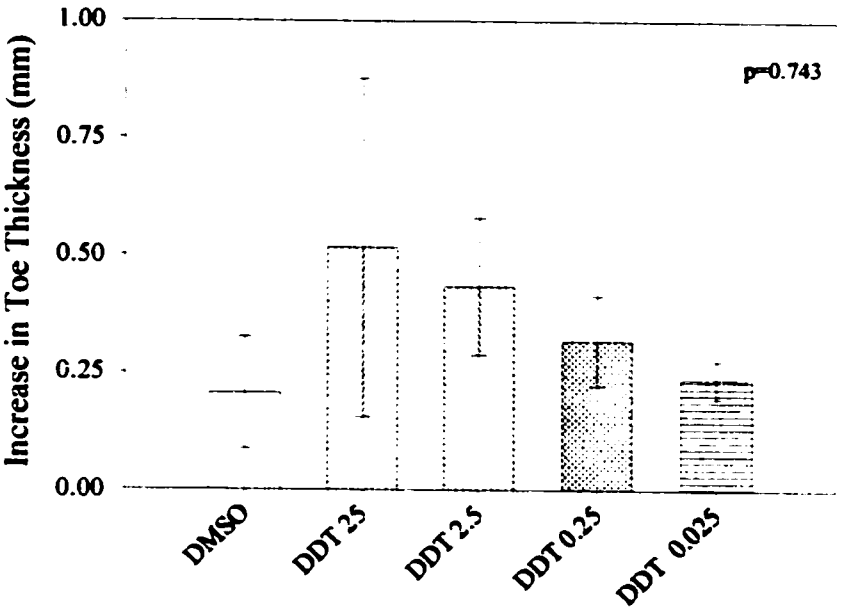

Figure 2.5 – Delayed type hypersensitivity as determined by change in toe thickness in response to PHA, in leopard frogs 2 weeks after exposure to stock solutions of 25, 2.5, 0.25, 0.025 and 0 mg/L of DDT in DMSO.

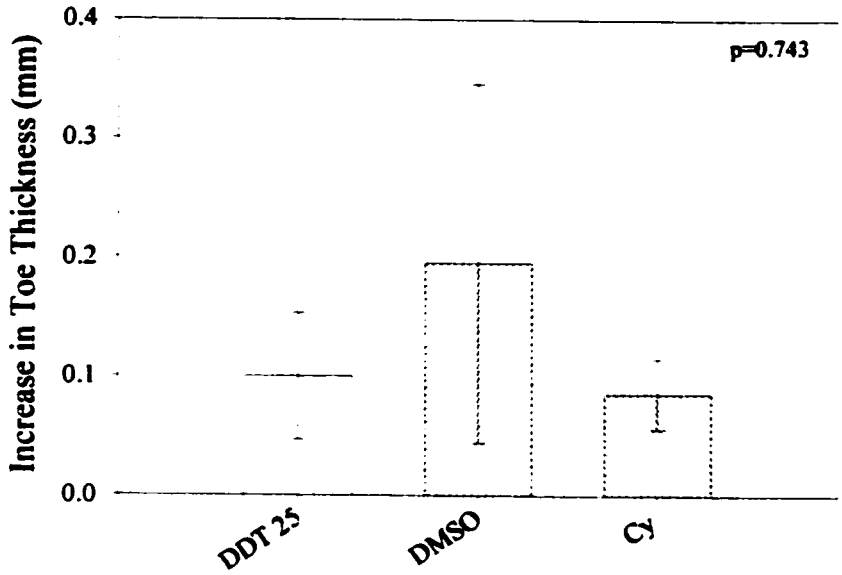

Figure 2.6 – DTH reaction to PHA-P in frogs immunized five days prior to contaminant exposure

2.3.3 Chemiluminescence Analysis of Zymozan Induced Oxidative Burst Products in Whole Blood

For the injection study, the mean peak CL values for week 8 are presented in Figure 2.7. The DMSO dosed frogs produced statistically more oxygen radicals than all other groups. The malathion and dieldrin frogs were significantly lower than the DDT or Cy exposed frogs.

Conflicting results occurred in the CL response after exposure to one pesticide, DDT, in the dose response study are shown if Figure 2.8. Initially the higher dosed groups (25 and 2.5 mg/L DDT stock solution) showed elevated response to the lower dose groups (0.25 and 0.025 mg/L DDT stock solution) and the DMSO control group. At the first time point, 2 weeks after exposure, the peak CL response of the high dose groups (25 and 2.5 mg/L DDT stock) is statistically ($p=0.012$ and 0.041 respectively) higher than that of the lower dose groups (0.25 and 0.025 mg/L DDT stock). The two lower dosed groups displayed a slightly higher reaction than the DMSO dosed (control) animals, although it was not statistically significant ($p=1.0$). Four weeks after exposure the highest exposure group (25 mg/L DDT stock solution) remained elevated in comparison to the other dosed groups (<0.0001). At this time point the DMSO control group showed a peak CL value that was statistically similar to the groups that had received 2.5 and 0.25 mg/L of DDT stock solution ($p=1.0$). Anomalously, the lowest exposure group (0.025 mg/L DDT stock solution) had a peak CL value which was statistically higher than DMSO, 2.5 and 0.25, ($p= 0.002$), but lower than the highest dose group (25 mg/L stock solution). At the time point eight weeks after exposure the highest dose group (25 mg/L stock solution) is at the same level as the DMSO controls but the

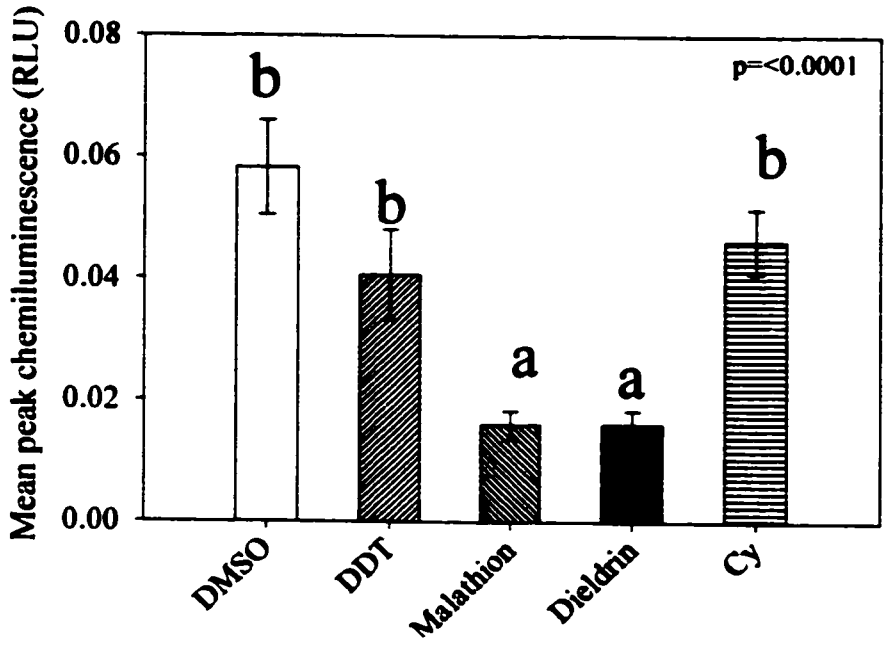

Figure 2.7 – Zymozan induced-chemiluminescence in leopard frogs 8 weeks after exposure to DMSO, DDT, dieldrin, malathion and Cy.

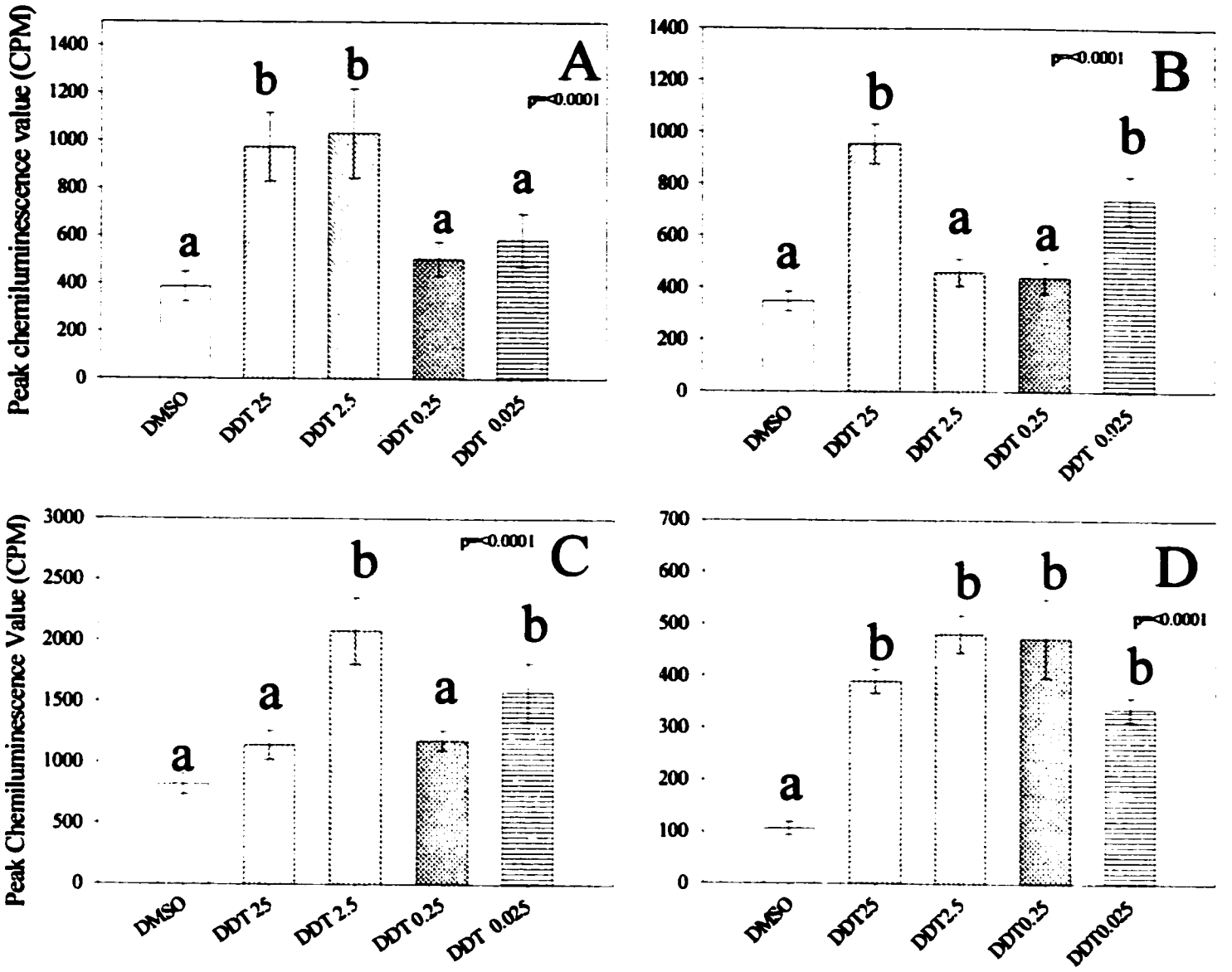

Figure 2.8 – Zymozan induced-chemiluminescence in leopard frogs A) 2 weeks, B) 4 weeks, C) 8 weeks and D) 12 weeks after exposure to stock solutions of 25, 2.5, 0.25, 0.025 and 0 mg/L of DDT in DMSO, n=5 per group

2.5 mg/L group is elevated ($p=<0.0001$). In comparison to the peak value at 2, 4 and 12 weeks, the reaction of all groups at 8 weeks is elevated. When the values of all the weeks are plotted together on a time course (Figure 2.9) a sharp increase is evident at week 8. This is likely indicative of an error in the assay rather than a change in response. At week 10 all groups are statistically elevated in comparison to the DMSO control group ($p=<0.0001$) and no difference is evident between the contaminant exposed groups. The peak CL values for the frogs in the reverse immunisation experiment are shown in Figure 2.10. No statistical differences are apparent between the groups two weeks after immunisation ($p=0.05$), although the Cy group is slightly higher. Four weeks after immunisation, the response of the Cy group and the DDT group is higher than that of the DMSO control group. The only statistical difference, however, exists between the DMSO group and the Cy group ($p=0.016$).

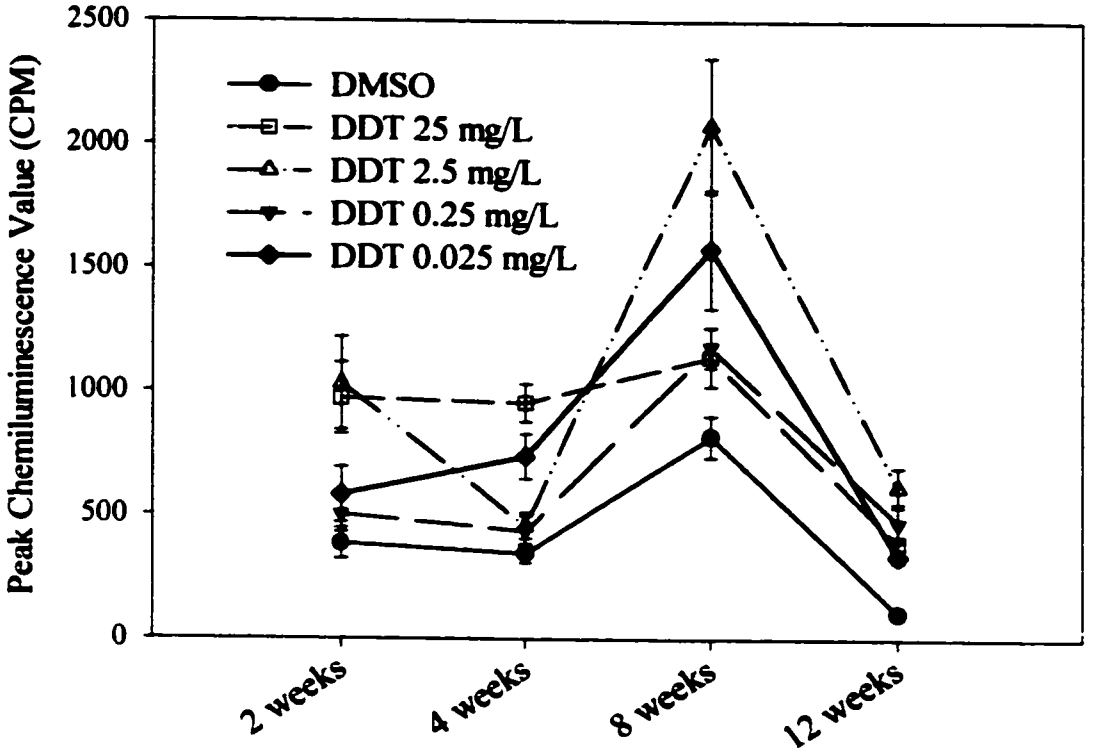

Figure 2.9 – Peak chemiluminescence values for all exposure groups during the dose response study

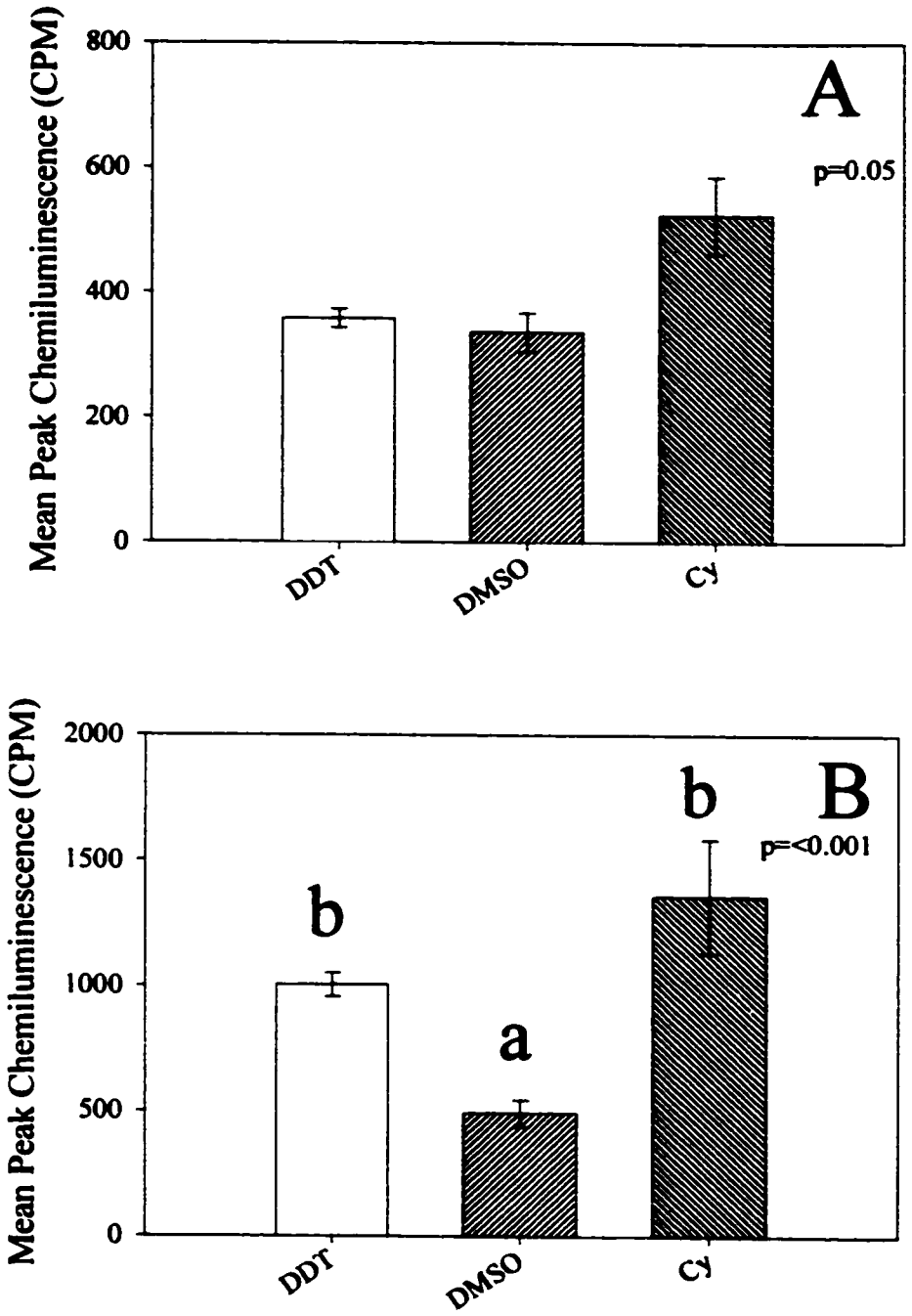

Figure 2.10 - Zymozan induced peak chemiluminescence in the reverse order immunization experiment A) 2 weeks and B) 4 weeks after immunization

2.3.4 Contaminant Analysis

For both the injection study and the dose response study, whole frog contaminant analysis was carried out on one frog from each exposure group on the second day after injection with the relevant compound to confirm that the dose injected was received. The results of the analysis for the injection study are shown in Table 2.1. Analyses were carried out on organochlorine compounds only, the concentration of the organophosphates, malathion and cyclophsphamide were not analysed. The expected concentration of DDT at two days was 750 ng/g of frog (Table 2.1). In actual fact the amount of DDT that was present in the tissue of the frog sacrificed two days after exposure was 923 ng/g of frog. The levels found in dieldrin were lower than the intended dose. The expected tissue concentration was 75 ng/g of frog, but the actual concentration was only 24 ng/g of frog.

Contaminant analysis was undertaken on five frogs from each group at week 8 and week 20 following contaminant exposure. The results of this are shown in Figure 2.11. It can be seen from this that there was statistically no difference between the level of either DDT or dieldrin within the exposed frogs between 8 and 20 weeks. A drop in concentration did occur from the frogs that were sacrificed 2 days after exposure and those which were maintained for 8 and 20 weeks.

During the injection study the weights were found to increase in all frogs throughout the duration of the experiment. Eight weeks after exposure the weight of the males averaged 43.6 g (±6.7g) and the females 64g (±10 g). Twenty weeks after exposure the average weight for males was 50.57g (±9 g) and for females the mean was 67.6 g (±11g). The weight increase over the initial 8 week experimental period was

CONTAMINANT GROUP	STOCK SOLUTION CONCENTRATION (mg/L)	EXPECTED CONCENTRATION ng/g FROG	ACTUAL CONCENTRATION ng/g FROG
DDT	250	750	923
Dieldrin	25	75	24
Malathion	330	990	NA
Cyclophsphamide	3000	9000	NA

Table 2.1– The concentration of the stock solution of the pesticides and Cy in DMSO used in the injection study and the expected and actual concentrations of DDT and dieldrin found in the frogs two days after exposure (n=1).

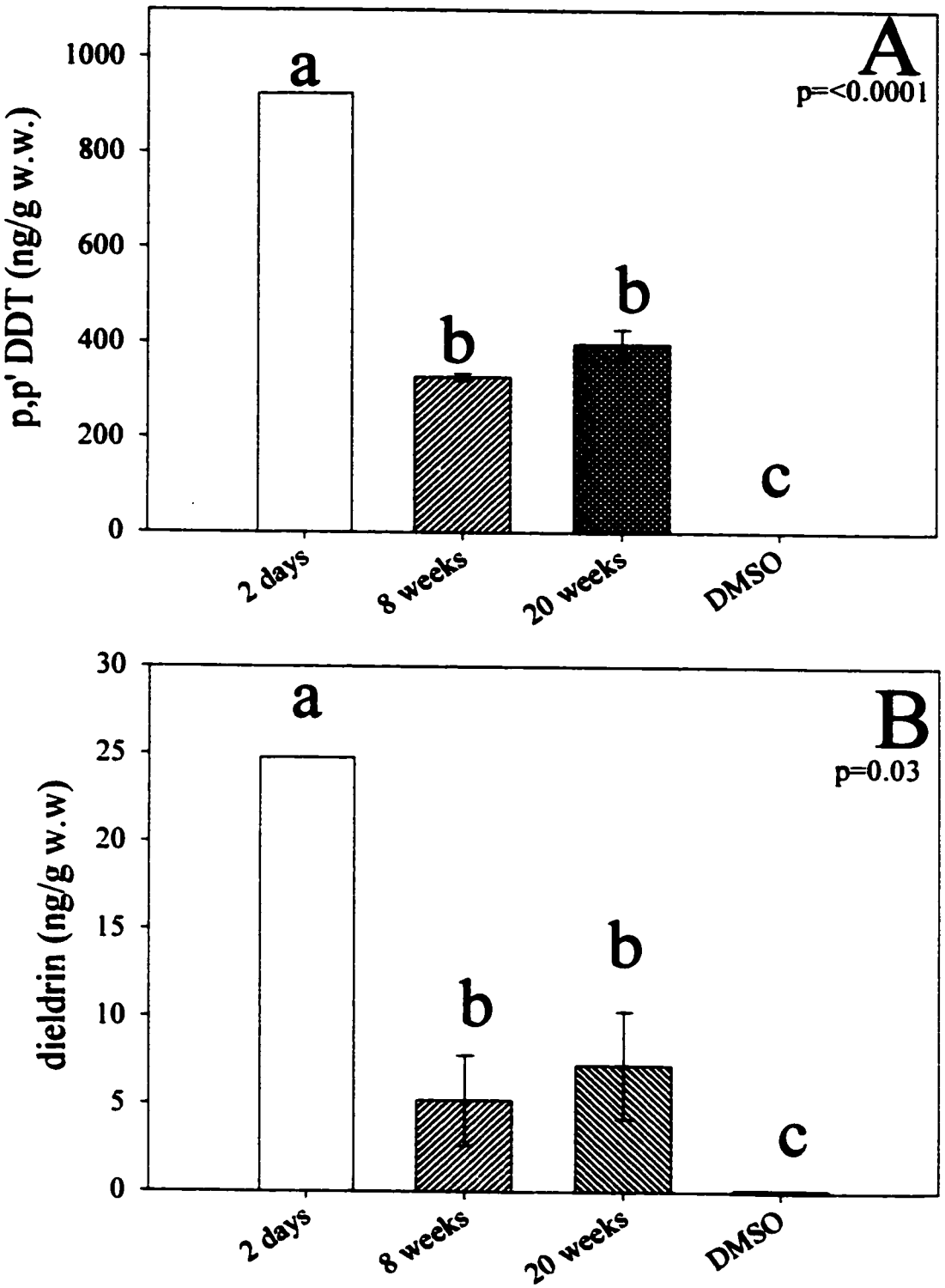

Figure 2.11 – Concentration of A) DDT and B) dieldrin in injection study frogs 2 days (n=1), 8 weeks (n=5) and 20 weeks (n=5) after exposure

10.38 g (±5.8g) for males and 11g (±7 g) for females. The increase at the 20 week period was 11.6 g (±9.4 g) for the males and 15.3 g (±7.7 g) for the females. The mean lipid content of frogs at 8 weeks was 3.97%(±1.49%) and at 20 weeks was 4.31% (±0.69%).

The concentration of the stock solutions used in the dose response, the expected concentration and the actual concentration found in frogs analysed 2 days after exposure are shown in Table 2.2. It can be seen from this that the expected concentration in each case is slightly lower than the actual concentration, though in all cases the values are similar. Although no DMSO frogs were analysed, previous analysis performed during the injection study. The background levels were about 0.66 ng/g which was similar to the concentrations used for the group exposed to 0.25 mg/mL stock solution.

The concentrations of DDT found in the three highest exposure groups (25, 2.5 and 0.25 mg/L) are shown in Figure 2.12. When the values are $\log_{10}$ transformed the relationship between the amount of DDT in the frog tissue two days after exposure is linear (r^2=0.9923). Contaminant analysis was not undertaken on the frogs from the reverse immunisation experiment but these frogs were dosed at the same time as the frogs in the dose response experiment, using the identical dosing methods and the same stock solutions.

CONTAMINANT GROUP	STOCK SOLUTION CONCENTRATION (mg/L)	EXPECTED CONCENTRATION ng/g FROG	ACTUAL CONCENTRATION ng/g FROG
DDT 25	25	75	84
DDT 2.5	2.5	7.5	8.2
DDT 0.25	0.25	0.75	0.78
DDT 0.025	0.025	0.075	N/A

Table 2.2 – The concentration of the stock solution of the DDT in DMSO used in the dose response study and the expected and actual concentrations of DDT found in the frogs two days after exposure (n=1).

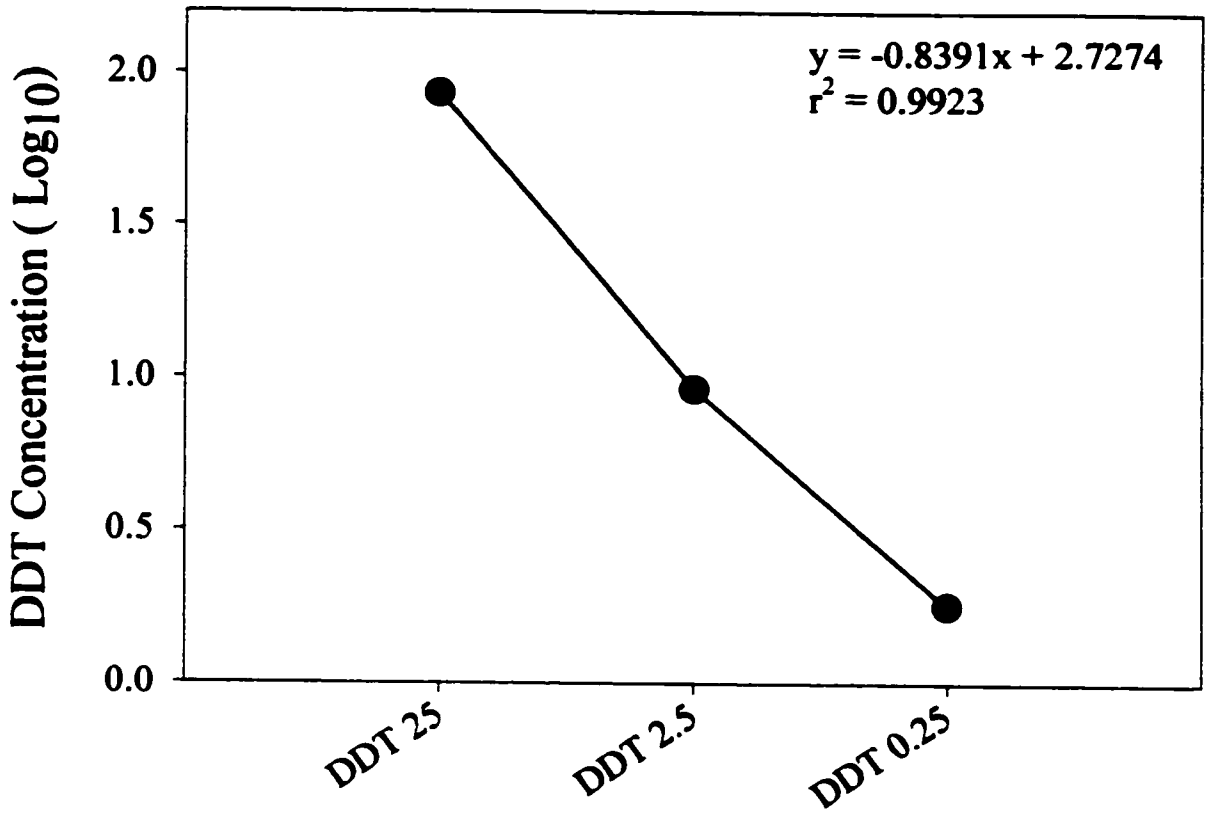

Figure 2.12 – Dose curve for DDT exposed frogs during the dose response study 2 days after exposure (n=1 per group)

2.4 DISCUSSION

The present investigation demonstrated that exposure to a sublethal dose of certain pesticides altered some aspects of the immune function of leopard frogs. The results from the injection study indicate that exposure to a single sublethal dose of DDT, malathion or dieldrin dramatically altered the production of KLH specific IgM when assayed two weeks after exposure. Gradual recovery of the IgM levels were apparent from week four to eight, and complete recovery to the levels in the control group was apparent by week twenty. The chemiluminescence assay in this experiment was only undertaken the time point eight weeks after exposure and at this time point the DMSO controls had a higher reaction that the pesticide groups. Interpretation of this result is difficult, however, as the assay was not introduced until later so the early time points are missing for comparative purposes. This assay is indicative of an innate endpoint, the killing of microbial pathogens is the result of the oxidative burst in phagocytes [57], which occurs early in the immune response, and the differences at 8 weeks may not be related to pesticide exposure. This method is thought to be relatively reliable tool to study the metabolic responses of phagocytes previously exposed to chemicals [57]. The whole blood chemiluminescence in *Rana sp.* mainly reflects neutrophil activity, since they are the most abundant blood phagocytes, but eosinophils and monocytes are also represented [58]. The results of the DTH experiment indicate that the pesticide exposed groups are reacting to a greater degree than the DMSO control group. It is possible that this might be a hypersensitivity reaction and could be a compensatory reaction to the loss of antibody production in these frogs.

The dose response study showed that even at lower concentrations DDT still affected the production of IgM in the exposed groups. At the concentrations used in the experiment however this suppression was not dose dependant. The CL reaction 2 weeks after exposure showed an elevated response in the two higher exposure groups (25 and 2.5 mg/L stock) in comparison to the DMSO control group and the lower exposure groups. Four weeks after exposure, the highest exposure group (25 mg/L) is still showing elevation. The results from week 8 are all anomalously high and this is probably indicative of an assay error and the results from this time point can not be considered reliable. Ten weeks after exposure, all of the contaminant groups have a higher response than the control group. At this time point the initial innate reaction is likely over and the response may not be due to the DDT exposure. The DTH reaction in this study showed a similar pattern to that of the injection study, although the results are also not significant. This could be indicative of a heightened T-cell reaction but the error might be masking the results. More information about the action of the B and T cells should be examined in a lymphocyte proliferation assay in the future to determine which mitogens are affecting which cell groups.

The reverse order immunisation experiment showed that when the frogs were immunised prior to pesticide exposure they no longer exhibited the suppression of IgM. The results for the CL and DTH also showed little difference between the exposure groups, except DMSO was elevated in comparison to the other groups at 4 weeks after exposure. This could suggest that if frogs were to come into contact with a challenge and were able to develop an immune reaction prior to pesticide exposure, their antibody response might not be altered by the exposure.

Contaminant analysis revealed that there was little change in the levels of DDT or dieldrin between weeks 8 – 20. The high concentrations used in the dose response study (DDT 750 ng/g and dieldrin 75 ng/g) were supposed to have a dramatic impact on the immune function to determine whether the immunological tools developed could measure those differences. The results of the chemical analysis at two days suggested that the dose received by the frogs was in the same range as that which was intended. Doses were given using disposable ½ mL syringes, a more appropriate method might be to use 50 – 100 µl calibrated glass syringes. The analysis at this time point used only one individual per group due to a lack of test subjects. This means that the confirmation of the dose is not as accurate as it would be if a larger group had been used. A significant difference is apparent between the levels of both DDT and dieldrin between initial concentration and week 8 levels. This could mean that the partial recovery in immune function of all groups by week eight is reflecting this initial drop. By week 20 the immune function has recovered but the contaminant levels have not changed at all. This means that the recovery of the immune system was not a function of depletion in contaminant levels. DDT and dieldrin levels between week 8 and 20 no longer impaired immune function, or the frogs physiological system was able to readjust to the presence of this stressor. The lower DDT levels used in the dose response study were confirmed when the two day contaminant frog for the three highest exposure groups (25, 2.5, 0.25 mg/L stock) were analysed. The relationship between the groups was linear and within the intended range. The lowest exposure groups displayed a similar concentration to that of the DMSO control frogs. At two weeks after exposure these lower concentrations did alter the immune function of the affected frogs. The recovery appeared to be much quicker than

in the injection study, possibly reflecting the lower doses used. A surprising enhancement of the peak CL levels occurred at week ten in all exposure groups. It is possible that this is reflecting the production of metabolites of DDT, such as DDE and DDD, which are known to be more immunotoxic then the parent compound and might stimulate activity in innate parameters at this late time point.

The findings in this study, particularly the response to DDT, do not always agree with previously published literature in other species. Our study has found that injected DDT did significantly effect alter the antibody production of exposed frogs when they were exposed prior to immunisation. There may be interspecies differences in immune response to particular compounds. For example hexachlorobenzene is strongly immunosuppressive in mice but cause stimulation in rats [112]. Previous studies examining the effect of DDT to humoral immune function in several species using a variety of functional parameters have shown suppression, enhancement or no change in response to antigen [113]. For example in rabbits [62] mice [63] and chickens [62] no effect on antibody titer to an oral dose of DDT was recorded. In contrast, Banjeree *et al.*, found that exposure to DDT at concentrations up to 200 ppm did suppress the IgM and IgG antibody production of mice [114]. This group also found that the metabolites of DDT, DDD and DDE were more immunosuppressive than the parent compound. At concentrations up to 100 ppm DDT exposure did not affect the primary antibody production of mice, but exposure in conjunction with single or multiple stressors did significantly alter the primary humoral response [115]. This suggests that the combination of xenobiotic factors and stress could influence immune competence.

Malathion is known to stimulate macrophage activity and the primary humoral-mediated response in mammals.[61]. In contrast the frogs injected with malathion showed a reduction of peak CL values at week 8 and a reduction in humoral antibody response. Technical grade malathion is contaminated with O,O,S,-trimethyl phosphorothioate (OOS-TMP) [61]. OOS-TMP has been found to block antibody response, in mice, although the effects were reversible [75]. The malathion used in this study was field grade so almost certainly contained OOS-TMP. This raises the issue of what compound is responsible for the suppressive effects, the malathion or OOS-TMP? This cannot be answered without further chemical analysis and the initiation of a study using OOS-TNP free malalthion.

Dieldrin has been used in several immunotoxicological studies on numerous species and has been found to reduce the number of antibody-producing cells and antibody production in mice [66, 67]. Reduction in IgM antibody titers in exposed frogs revealed that dieldrin altered the production of antibodies, but in contrast to mammalian systems, to a lesser extent than did DDT, malathion or Cy.

DTH reactions are known to be reliable correlates of cell-mediated immunity, but they are less sensitive than *in vitro* methods [57]. DTH reactions require the specific recognition of the antigen by activated T-lymphocytes, which subsequently proliferate and release cytokines. These cytokines increase vascular permeability, induce vasodilatation and macrophage accumulation, and finally antigen destruction [57]. The standard error in this assay was high and dependant upon a variety of factors. The small size of the frog introduces numerous difficulties when trying to establish measurement differences in toe thickness. Measurements were done using an electronic microcalliper,

to determine the thickness of the middle toe of the hind foot of each frog. This thickness was thought to be indicative of the proliferation of activated T-cells to the area. Many sources of error can be identified such as the area of measurement, the injection of PHA-P into the site and discrepancies by the calliper operator. Efforts were made to reduce these errors by taking repeated measurements of the toe, using a dissecting lamp to magnify the area and having only one person do all the measurements. The measuring technique used in this study was not sensitive enough to register the slight changes that occurred. If a more sensitive method could be developed this assay could prove to be a reliable non-sacrificial immunological marker.

In this study, although frequently not significant, the overwhelming trend showed that pesticides enhanced the DTH reaction in the exposed frogs in comparison to the DMSO control group. Past studies using skin response to a stimulatory compound and correlating this to contaminant exposure has found differing results. For example Ross *et al* found that seals fed contaminated Baltic herring exhibited a suppressed delayed-type hypersensitivity response to immunisation with ovalbumin in comparison to seals fed less contaminated Altantic herring [92]. Similarly Grasman et al used a phytohemagglutinin skin test to determine whether contaminant- associated immunosuppression occured in prefledgling gulls and terns from the Great Lakes. They found that in both species, there was a strong exposure-response relationship between organochlorines and suppressed T-cell-mediated immunity [93]. In mice the DTH reaction of DDT exposed mice was suppressed [114]. The opposite scenario has been exhibited in some species with exposure to contaminants producing an increased skin reaction. Pesticide exposure appeared to cause an increase in the toe skin scratch reaction in response to PHA-P

injection in all frog exposure groups. Cellular immunity is reported to be enhanced by DDT exposure [113] and this might explain the elevated DTH reactions in the injection study and the dose response study. This could be a compensatory reaction to the loss of antibody production in these frogs. Consequences related to a loss of self tolerance, or a loss of normal regulatory or compensatory mechanisms, may include hypersensitivity or autoimmunity [61, 116].

2.5 CONCLUSIONS

The results of the injection study indicate that pesticides, at a high dose do alter the immune function of the leopard frog. These alterations are measurable but further development of assays, is need to quantify these changes with greater accuracy. For example introduction of a specific *Rana* antibody for the ELISA assay, greater measurement accuracy for the DTH and further optimisation of the CL would be warranted. At the lower concentrations used in the dose response study, DDT continued to show alterations in the immune function. The order of immunisation was found to be an important factor as the modification in immune function disappeared when frogs were able to establish antibody production prior to toxicological challenge. Some changes in frog immune function responded in a different manner to mammalian species tested. The differences in assay type and experimental procedure between studies varied, making comparisons difficult. These results indicate that in terms of immune alterations, amphibians may react differently to pesticide exposure than mammals. This could have serious implications for amphibian populations if regulatory authorities determine safe levels based only on mammalian model toxicity endpoints. Sublethal alterations occur in

response to pesticides and further work using field based techniques is warranted to further evaluate whether pesticides have a role in increasing the incidence of disease in stressed frog populations.

CHAPTER 3 .0 – APPLICATION OF IMMUNOLOGICAL METHODS; DIFFERENCES IN IMMUNE FUNCTION IN ONTARIO FIELD COLLECTED NORTHERN LEOPARD FROGS (*Rana pipiens*)

3.1 INTRODUCTION

Organochlorine insecticides are known to significantly alter response to antigenic stimuli of the immune system [87]. The ecology and physiology of amphibians may expose them to a wide variety of routes of contamination [16]. In addition, as a result of their thin, permeable skins and prolonged exposure, first in their aquatic and then in their terrestrial life stages, amphibians are thought to be susceptible to xenobiotics in their environment [18]. Knowledge of pesticide effects on amphibians is largely limited to short term toxicity tests conducted under highly artificial conditions to determine the lethal concentration (LC_{50}) for 50% of the population within a given time. This time period is often very short (1-4 days) [41] and does not consider more subtle effects such as alterations in immune capacity. It is plausible that pesticide exposure may be adversely effecting the immune function of exposed populations making them more susceptible to a variety of infections.

There are a variety of physical and behavioural aspects that might make amphibians good indicators of contaminant stress. For example, amphibians typically have a small home range and are relatively long lived. These factors result in an excellent system to develop *in situ* cause and effect models for toxic chemicals [27]. In addition they live in small, temporary ponds and forested areas, which are often targets of aerial pesticide sprays [22, 23] and subject to agricultural field or urban runoff, [24].

In Ontario the leopard frog is reportedly widespread and abundant throughout the southern and central regions, though they are sparse in northern regions where local declines appear to have occurred [117]. A recent study examining pond communities in Southwestern Ontario noted that anecdotal reports suggested that *R. pipiens* were the most abundant frog in the Essex plain, but that during the study (1992-1993) they declined in occurrence in all regions [118].

Organochlorine pesticide use in North America has decreased since the 1970s, but pesticides still pose a threat to biota because of their toxicity, environmental persistence, and potential to bioaccumulate in foodchains [36]. A recent study examining the disappearance of the threatened California red-legged frog (*Rana aurora draytonia*), found it to have a strong positive association with the percentage of agricultural land use upwind [104]. The historic application of DDT to wetlands for mosquito control may be of great importance in affecting amphibian populations [105]. Essex County is one of the premier agricultural areas of Canada. The soils and climate are particularly suited to agriculture. A wide variety of cash crops (i.e. tomatoes, strawberries, lettuce, fruit, etc.) are grown, as well as wheat, corn and soybeans. Much of the southeastern portion of the county also supports greenhouses. This agricultural use means that it is subject to large amounts of pesticide use. Since 1972 three species of amphibians have become locally extinct in Essex county, and a fourth species has been reduced in abundance in Point Pelee National Park [36]. DDT and dieldrin have recently been found in the tissues of frogs from Point Pelee [36] at concentrations up to 160 μg/kg and 199 μg/kg respectively (see table in appendix A for more tissue concentrations).

Experimental studies in numerous species demonstrate that exposure to synthetic chemicals can result in increases or decreases in measured immune parameters and hypersensitivity [106]. In the past, toxicological studies in amphibians have focused primarily on collecting tissues to measure persistent contaminants [16]. In chapter 2 the effect of certain pesticides on innate, humoral and cell-mediated immunological endpoints in *Rana pipiens* was examined. It was found that the pesticides DDT, dieldrin and malathion (dose 750, 75 and 990 ng/g) had deleterious effects on amphibian immune function when a single sublethal dose was administered. This study also showed that at lower doses (75-7.5 ng/g) DDT could still effect immune function.

The objective of this study was to apply immunological techniques to northern leopard frogs *(Rana pipiens)* collected in different areas of Ontario to determine whether spatial variation in immune function existed. Organochlorine contaminants were evaluated to establish whether alterations in immune functions could be correlated to contaminant levels.

3.2 METHODS

3.2.1 Experimental Design

Northern leopard frogs *(Rana pipiens)* were collected from streams, ponds, marshes, grasslands and meadows. The sites are shown on Figure 3.1 and included two control sites and four sites in Essex county. The control sites included an equine facility

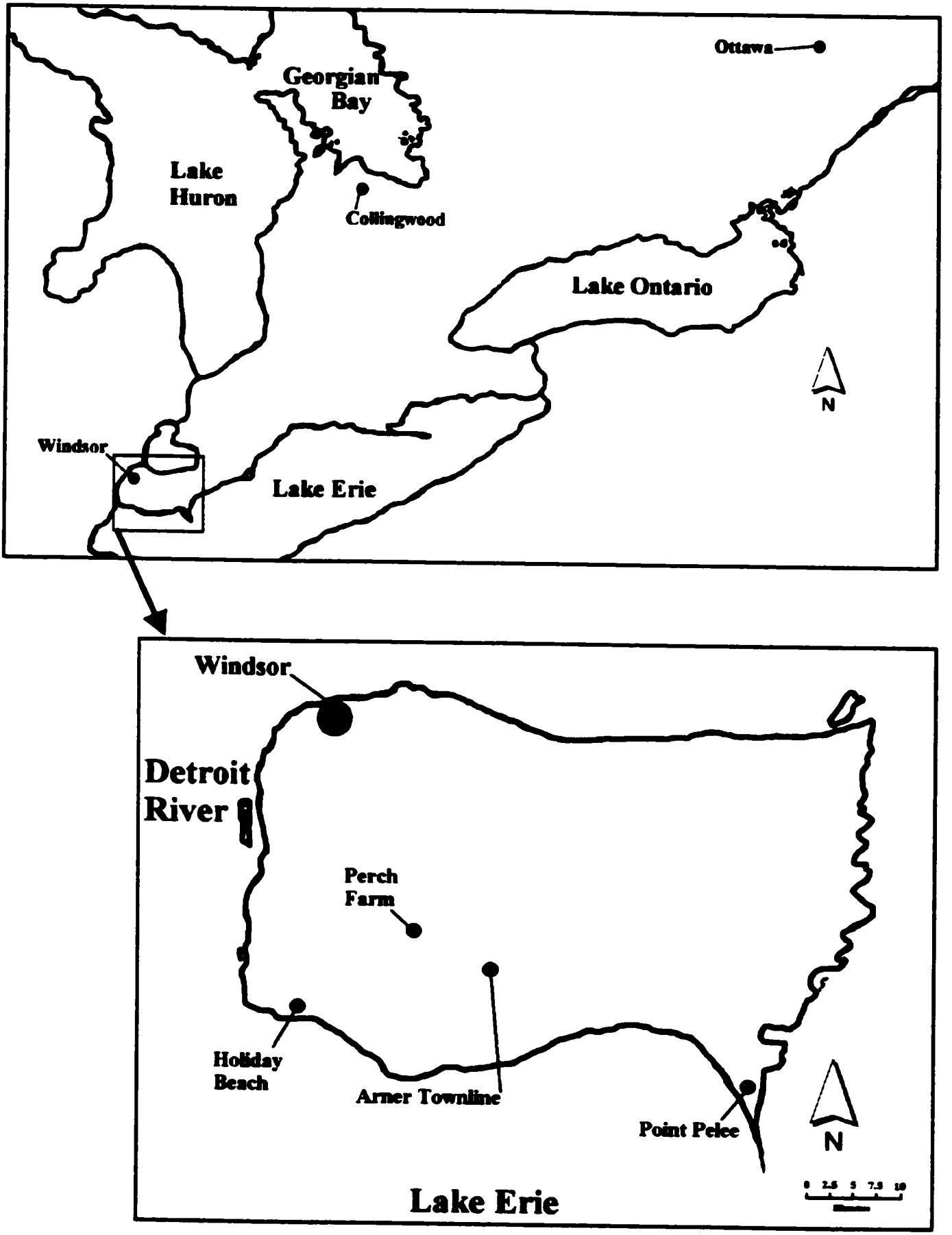

Figure 3.1 – Collection sites in Ontario *for Rana pipiens* in the autumn of 2000. The inset shows where the frogs were collected in Essex county

near Ottawa and a marsh near Hepworth, north of Collingwood. The sites in Essex County were a wooded lot near Arner Townline, a creek just north of Point Pelee, a perch farm near McGregor and a wetland area adjacent to Holiday Beach. Thirteen to fifteen frogs were collected at each site, ten to be used to determine immunological competence of the group and the remaining frogs sacrificed for contaminant analysis.

Collection of the frogs was undertaken with a large, fine mesh, long handled trout net, in the morning and evening. During collection the frogs were placed into small plastic carrying containers. Periodically this container was emptied into a large, communal container filled with several inches of water. The frogs from different sites were kept segregated at all times during transport. Efforts were made to ensure frogs did not overheat, and while in the field, handling was kept to a minimum to reduce stress.

Upon arrival at the Great Lakes Institute for Environmental Research (GLIER) the frogs were housed in aquaria (30 x 90 cm) in groups of five and provided with a washed concrete block covered in rubber matting as a feeding platform. Underwater charcoal filters (Quick~Filter® (802), Hagen) were utilised to decontaminate the water. The tanks were emptied and scrubbed once a week and refilled with chlorinated tap water. Frogs were fed daily with crickets that were maintained on a diet of bird granules (Tropican, Hagen, Inc, Montreal). Individuals were identified by the spot pattern on the back using a combination of close up photographs, line drawings and a detailed written description.

The frogs were kept for approximately one to three weeks to acclimate, recover from transport stress and allow for all collections to be completed. Collections from all sites were finished prior to the initiation of immune assays. Immunological assays were

undertaken within the first 4 weeks of capture. The initial assays used were antibody response to KLH, chemiluminescence and delayed-type hypersensitivity. Two months later, the antibody response to KLH-DNP and the chemiluminescence assays were repeated.

3.2.2 Immune Assays

Immunisation and blood collection were carried out as outlined in chapter 2, section 2.2.3 and 2.2.4 respectively. The frogs were boosted with KLH and Titer Max two months after capture. Two weeks after the initial immunisation, the frogs were bled, a CL reaction initiated on whole blood (see method chapter 2, section 2.2.7) and an ELISA initiated on frog plasma (method chapter 2, section 2.2.5). Nine days after immunisation the DTH assay was initiated (method chapter 2, section 2.2.6). The ELISA and CL were repeated two weeks after the second immunisation.

3.2.3 Contaminant Analysis

Between three and five frogs from each site were sacrificed for contaminant analysis upon arrival at GLIER and stored frozen (-20°C) in hexane rinsed foil. Sample preparation was undertaken using the same method as outlined in chapter 2, section 2.2.8.

3.2.4 Statistical Treatment

All statistics were performed using Systat version 7.0. Differences were determined using analysis of variance (ANOVA), and post hoc test Bonferroni's. Outliers were omitted according to Studentized residual values generated by Systat.

3.3.0 RESULTS

3.3.1 Antibody Response to KLH-DNP

When the antibody response to KLH-DNP was assayed, within the first month of capture, differences were apparent between the groups collected from varying regions of Ontario ($p=<0.0001$). These results are displayed in Figure 3.2. From this it can be seen that the frogs collected near Collingwood showed a dramatically higher antibody response which was statistically different from all other groups ($p=<0.0001$ in all cases). When expressed as a percentage of the Collingwood group the mean reactions were 35% for the Ottawa group, 20% for the group collected at the perch farm, 28% for the frogs from Arner Townline, 14% for the Holiday Beach group and 34% for the Point Pelee frogs. No statistical differences existed between these groups although the Holiday Beach frogs had the lowest antibody response.

When the antibody response assay was repeated two months later, the results were quite different although statistical differences still exist ($p=<0.0001$). The results at this time point are shown in Figure 3.33. All the groups are statistically similar except for Point Pelee, which shows an antibody response which is significantly lower ($p=<0.0001$). When expressed as a percentage of the Collingwood group, the frogs from the Ottawa region are at 80%, (60% increase), the Arner townline group are at 97% (69% increase), the perch farm group is at 98% (78% rise) and the Holiday Beach frogs are slightly lower with 66% (52% increase). The frogs from Point Pelee, on the other hand are at 27% (7% lower than their initial value).

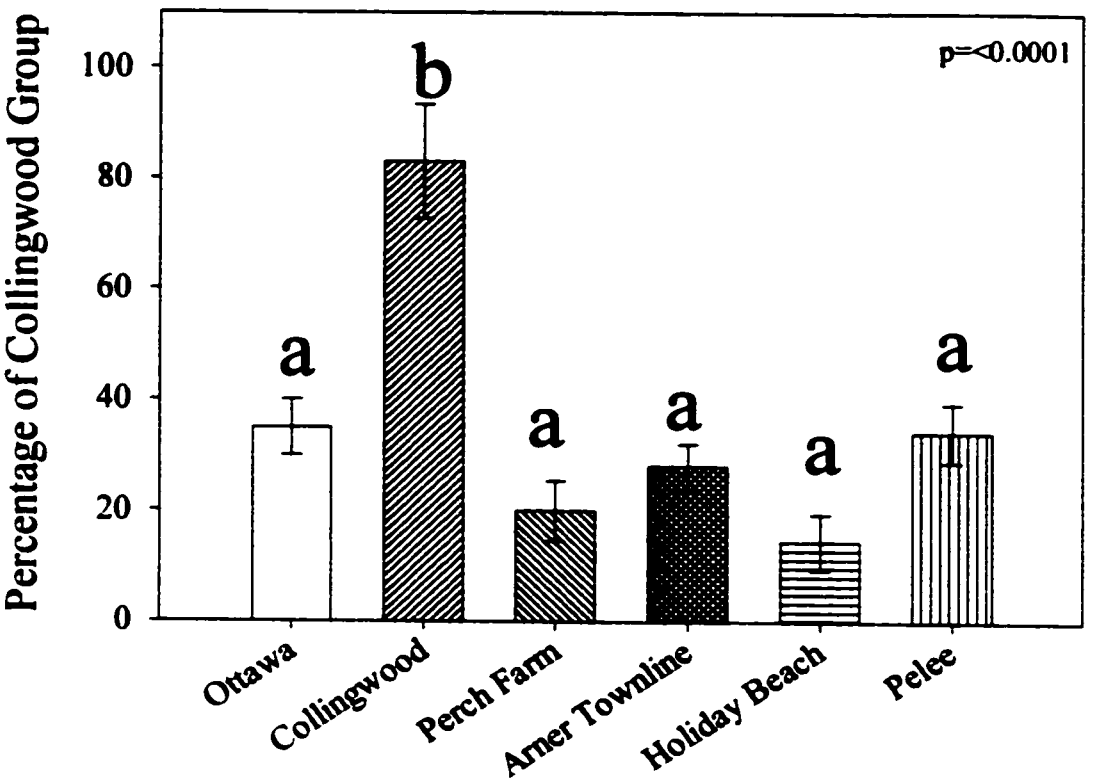

Figure 3.2 – Antibody response to KLH-DNP, assayed with the first month of capture, in field collected leopard frogs from the regions near Ottawa (n=8) and Collingwood (n=7) and Essex County; Perch Farm (n=7), Arner Townline (n=11), Holiday Beach (n=12), Pelee (n=12)

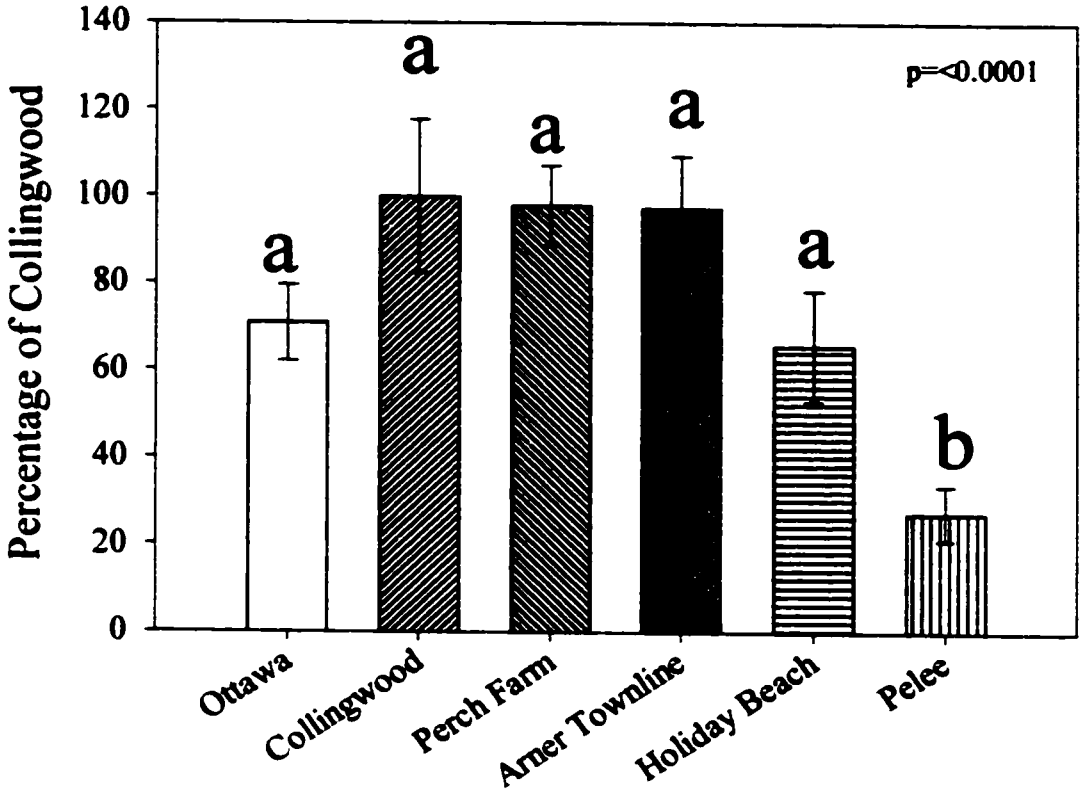

Figure 3.3 – Antibody response to KLH-DNP, assayed two months after initail assay, in field collected leopard frogs from the regions near Ottawa (n=8) and Collingwood (n=7) and Essex County; Perch Farm (n=6), Arner Townline (n=11), Holiday Beach (n=12), Pelee (n=12)

3.3.2 Chemiluminescence

When the frogs were assayed within the first month of capture the peak CL values showed differences ($p=<0.0001$) between the sites. Figure 3.4 shows the peak CL for this initial sampling occasion. In the initial CL assay the mean peak values (counts per minute) for Ottawa (0.0715 CPM), Collingwood (0.0593 CPM) and Pelee (0.0946 CPM) were higher than the other groups. The values for the Ottawa and Collingwood frogs were statistically similar, but showed differences in comparison to Point Pelee ($p=0.002$ and $p=<0.0001$), Arner ($p=<0.0001$ and $p=0.0003$), Holiday Beach ($p=<0.0001$) and the Perch farm ($p=<0.0001$). Point Pelee was different from all other groups ($p=<0.0001$). The mean peak CL values for the frogs collected near Holiday Beach (0.0290 CPM), Arner (0.0329 CPM) and the Perch farm (0.0227 CPM) were statistically similar.

When the same frogs were assayed, two months after the initial sampling period, the peak CL values for the groups had changed, although differences still existed between the groups ($p=<0.0001$). The results, two months after the initial assay, are shown in Figure 3.5. The statistics at this time point suggested overlap in the similarities of the groups, this is indicated by a letter in the Figure. At this point the frogs collected from near Holiday Beach had the highest reaction, with a mean peak CL of 0.1475 CPM which was statistically different from the frogs from Collingwood ($p=0.049$) (mean 0.0387) although this result is not highly significant. The mean peak CL at the Holiday Beach site was also not the same as at the Perch farm ($p=0.01$) (mean peak CL 0.0261 CPM) or Arner Townline ($p=<0.0001$) (mean peak CL 0.0315 CPM). The Point Pelee frogs (mean peak CL 0.0507 CPM) showed a peak CL that was statistically different from all the groups except the one from Ottawa (mean peak CL 0.0589 CPM). The mean peak CL in

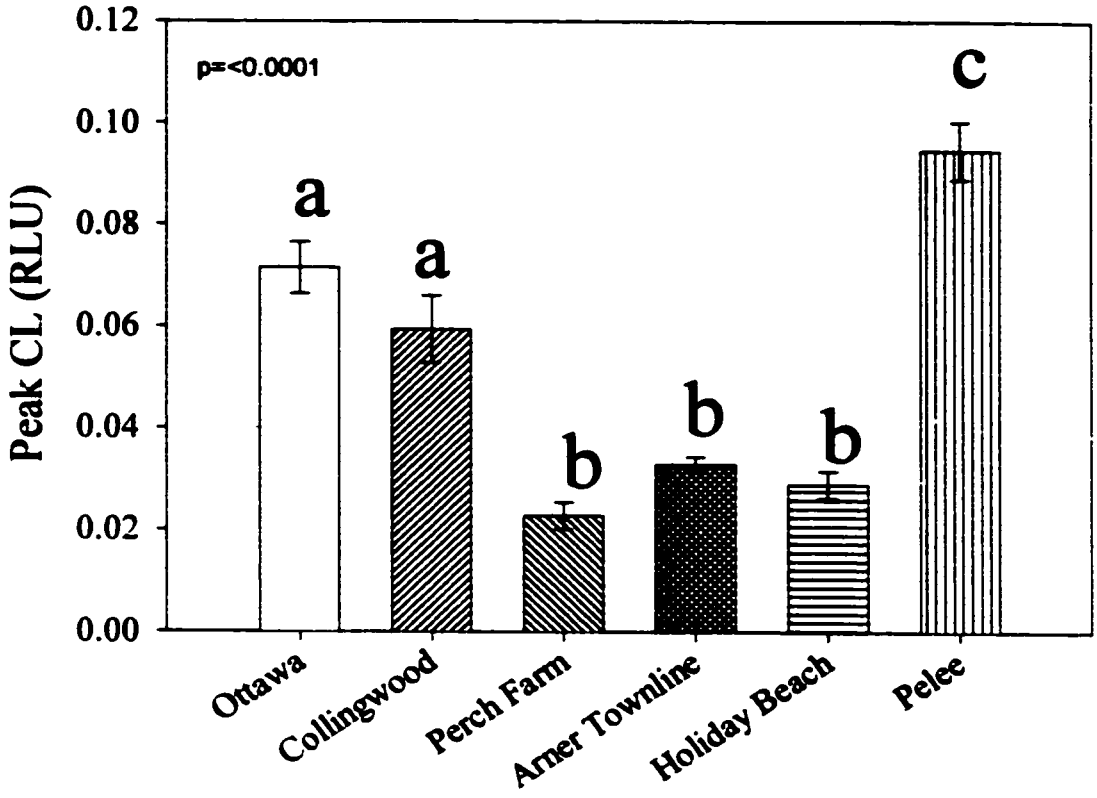

Figure 3.4– Peak chemiluminescence in whole blood, assayed with the first month of capture, in field collected leopard frogs from the regions near Ottawa (n=8) and Collingwood (n=7) and Essex County; Perch Farm (n=7), Arner Townline (n=11), Holiday Beach (n=12), Pelee (n=12)

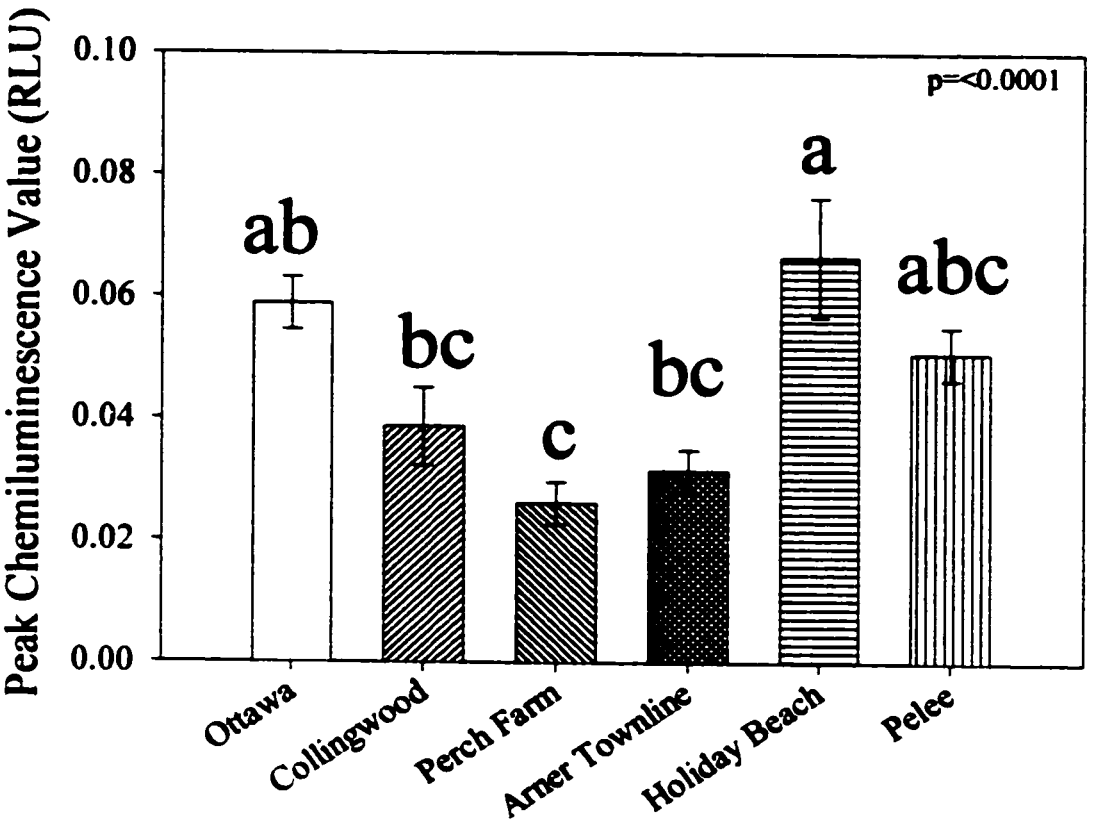

Figure 3.5 – Peak chemiluminescence in whole blood, two months after initial assay, in field collected leopard frogs from the regions near Ottawa (n=8) and Collingwood (n=7) and Essex County; Perch Farm (n=6), Arner Townline (n=11), Holiday Beach (n=12), Pelee (n=12)

the frogs from Ottawa was dissimilar to those from the Perch farm site (p=0.034) and Arner Townline (p=0.045).

3.3.3 Delayed-Type Hypersensitivity

The results for the DTH which was undertaken within the first month of capture, are shown in Figure 3.6. The frogs collected from Collingwood had the lowest reaction which is statistically different from that of the Arner Townline frogs (p=0.013) and the Holiday Beach group (p=0.044). The highest DTH reaction was seen in the Arner Towline frogs, followed closely by the frogs from Point Pelee.

3.3.4 Differences Between Antibody Response and CL at the Two Time Points

When all the assay values for the two time periods are plotted together for the antibody response and CL assays as shown in Figure 3.7 the changes between the groups become more evident. When the mean of the results from 2 months are subtracted from the values obtained for the initial assay, it is apparent that the peak CL values for Ottawa (-0.0126), Collingwood (-0.0207), Arner (-0.00138) and Pelee (-0.043939) have all decreased. The peak CL value for Holiday Beach increased dramatically (+0.118541) and the Perch Farm (+0.0014) rose only marginally.

For the antibody response assay it is clear that all the groups, except for Point Pelee displayed a similar response when kept in captivity for three to four months. The results for all the assays are displayed pictorially in Table 3.1.

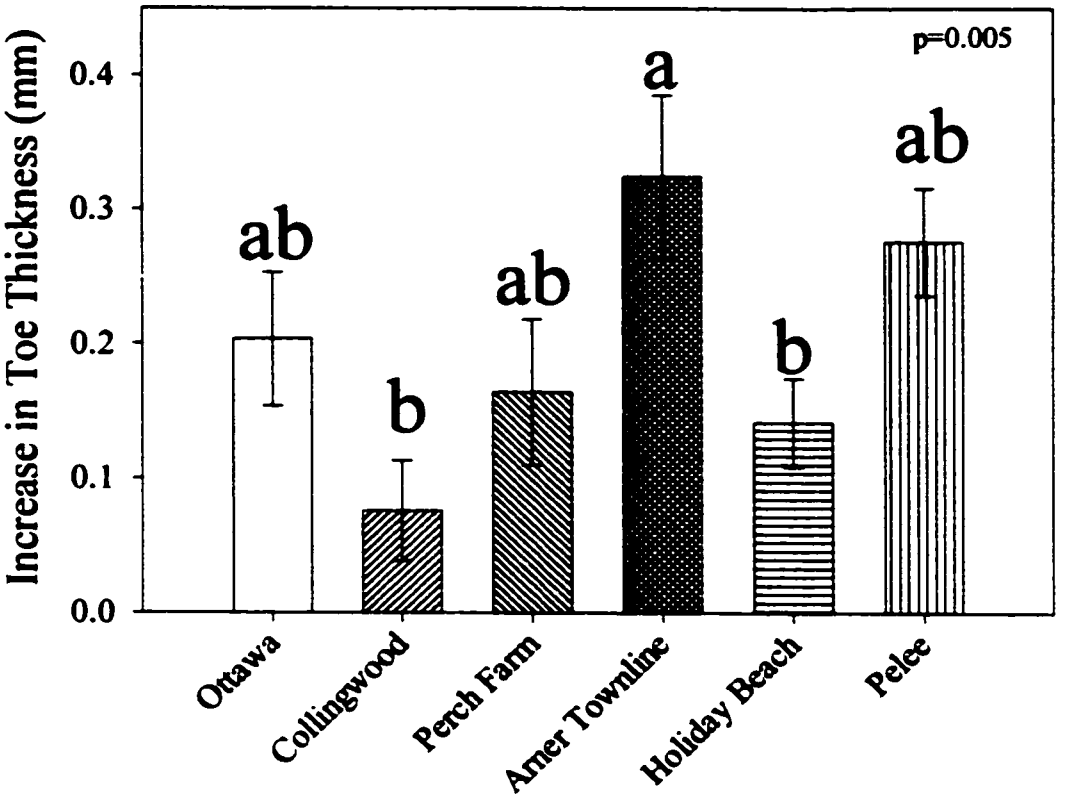

Figure 3.6 - Mean increase in toe thickness as an indication of DTH reaction in response to PHA-P at 24 hours in field collected leopard frogs from the regions near Ottawa (n=8) and Collingwood (n=7) and Essex County; Perch Farm (n=6), Arner Townline (n=11), Holiday Beach (n=12), Pelee (n=12)

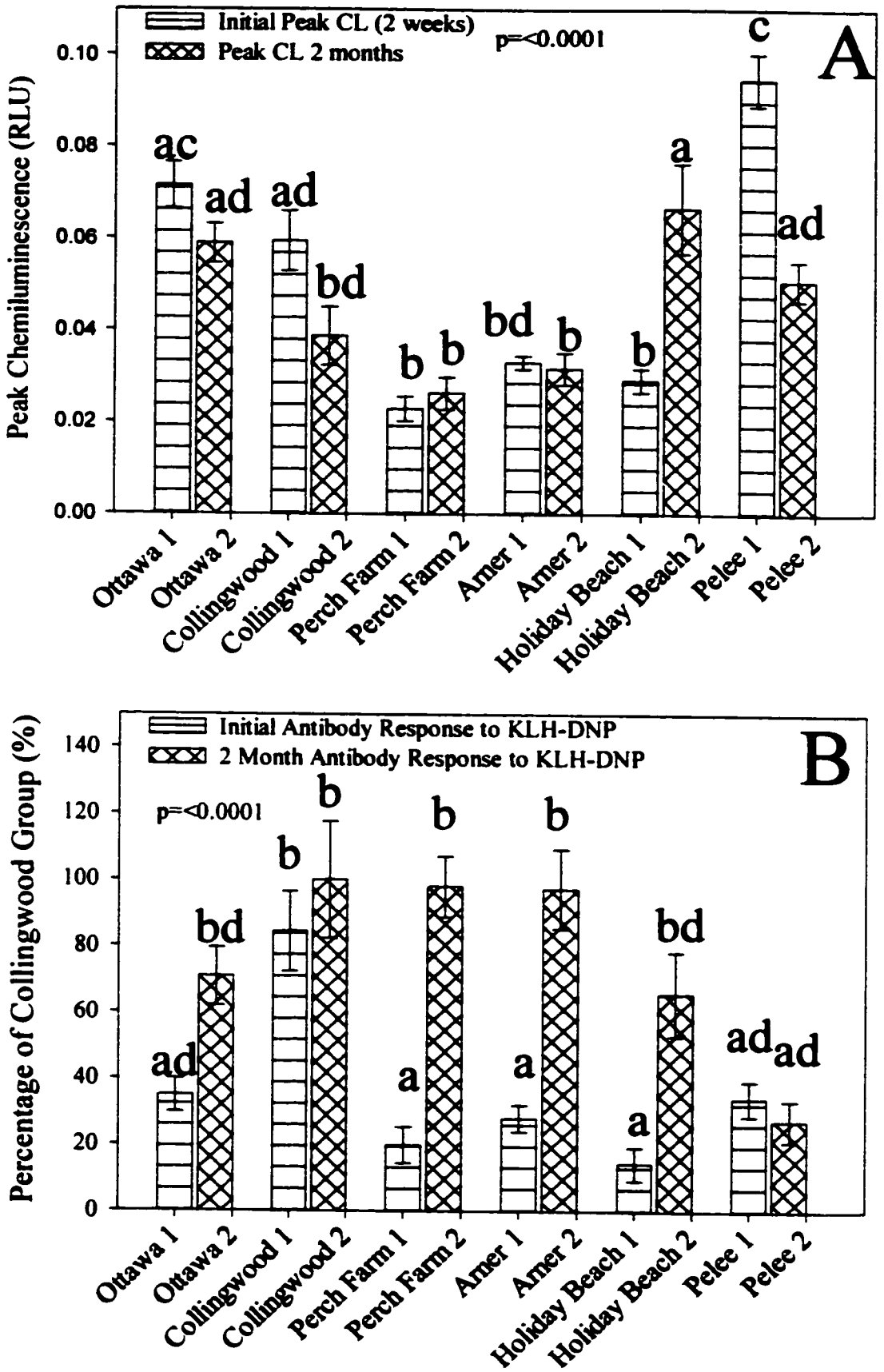

Figure 3.7 – A) Peak chemiluminescence in whole blood, for initial assay period and two months later and B) antibody response to KLH, expressed as a percentage of the Collingwood group, at initial assay period and two months later, in field collected leopard frogs from the regions near Ottawa (n=8) and Collingwood (n=7) and Essex County; Perch Farm (n=7), Arner Townline (n=11), Holiday Beach (n=12), Pelee (n=12)

↑ = High — = Medium ↓ = Low	ANTIBODY RESPONSE TO KLH		PEAK CHEMILUMINESCENCE		DTH
	Initial	Two months	Initial	Two months	Initial
Ottawa	↓	—	↑	—	—
Collingwood	↑	↑	↑	↓	↓
Perch Farm	↓	↑	↓	↓	—
Arner Townline	↓	↑	↓	↓	↑
Holiday Beach	↓	—	↓	↑	—
Pelee	↓	↓	↑	↑	↑

Table 3.1 – Summary of the mean immune results for all field collected leopard frogs from various regions of Ontario. The initial assay period and two months later are presented.

3.3.5 Contaminant Analysis

The results of the contaminant analysis are shown in Table 3.2 (full results are tabulated in appendix C and D). For some sites only two frogs were collected for contaminant analysis. One of the frogs from Point Pelee (Point Pelee 1) has elevated levels of DDT (121 ng/g) , DDD (17 ng/g) and DDE (491 ng/g). The other frog from Point Pelee displays high values, DDT (2.94 ng/g), and DDE (16 ng/g) relative to the values found at other sites, although it is much lower than Point Pelee frog 1. Dieldrin was also found to be very high in these frogs (23 and 21 ng/g respectively). Holiday Beach had the next highest levels of DDE (5.40 – 7.58 ng/g) although DDT and DDD were low or absent.

Dieldrin at this site was elevated slightly in one frog (3.3 ng/g). No other dramatic differences existed in the contaminant levels between the various groups. The mean lipids found in the frogs at all sites were found to be 1.678±0.55%. The mean increase in the weights of the frogs from all sites were roughly similar (Ottawa 2.84 ±2.7g, Perch Farm 2.7±2.5g, Holiday Beach 1.54±2.1 g and Point Pelee 1.98±2.8 g) with the exception of Arner Townline (7.86±8.6 g) and Collingwood frogs (5.76±3.3 g).

SAMPLE IDENTIFICATION	pp'-DDE ng/g/w.w.	p,p'-DDD ng/g/w.w	pp'-DDT ng/g/w.w	DIELDRIN ng/g/w.w
Point Pelee 1	491.4556	17.1168	121.0158	21.15503
Point Pelee 2	16.89451	0.199923	2.936114	28.28836
Holiday Beach 1	5.39376	0	0.511136	0.563529
Holiday Beach 2	6.435546	0.157564	0.953576	3.303739
Holiday Beach 2	7.565253	0.521531	1.066075	3.407389
Arner Townline 1	2.371169	0	0.355029	0.98746
Arner Townline 1	2.655069	0	0.415681	1.960223
Arner Townline 2	3.760703	0.294091	0.782184	2.35868
Perch Farm 1	1.448622	0.123362	0.26844	0.180211
Perch Farm 1	1.577122	0	0.445486	0.169506
Perch Farm 2	1.352868	0	0	0.156543
Perch Farm 3	1.276187	0	0	0.244057
Collingwood 1	2.216554	0	0.152659	0.277424
Collingwood 2	2.91542	0	0.81863	0.345237
Collingwood 5	2.273082	0	0	0.121975
Ottawa 3	1.005775	0	0.254993	0.173463
Ottawa 4	0.77649	0	0	0.114476
Ottawa 5	1.091571	0	0	0.196554

Table 3.2 – A selection of the results of contaminant analysis of frogs collected from various areas of Ontario

3.4 DISCUSSION

Spatial differences in aspects of immune function were apparent between the frogs collected from different areas of Ontario. When the results of the immune assays are examined together, as shown in Table 3.1, the changes at the different time points can be seen.

3.4.1 Antibody Response to KLH-DNP

When the antibody response to KLH was examined within the first month after capture clear spatial differences were apparent. The Collingwood group had a reaction that was dramatically higher than that of all the other frog groups tested. No statistical differences in antibody response were apparent between the frogs collected from Ottawa or regions in Essex County at this time point. When the same assay was repeated two months later the antibody response of the groups had changed dramatically and all the groups except Point Pelee were responding to the same level as the Collingwood group. This assay is a measure of the humoral response of the frogs to a protein stimulus. The results suggest shows when the frogs were first captured, the ability of the different populations to produce antibodies was impaired in all the groups, when compared to the response of the Collingwood group. The plot of all values together (Figure 3.7 B), shows that the antibody titer of the frogs collected from the Collingwood area has not changed between the two testing periods, but all other groups, except Pelee have increased to a similar level. In light of the phenomenally high DDT, DDD, DDE and dieldrin values found in the tissue of one of the Point Pelee frogs it is possible that this reduction was reflecting impaired immune function as a result of contaminant burdens.

3.4.2 Delayed-Type Hypersensitivity

The DTH assay followed a similar pattern to the results found in the studies in chapter 2. The Collingwood group had a reaction to PHA-P which was statistically lower than all of the other groups. This is in line with the findings for the antibody response that found that frogs from Collingwood had a higher reaction to all of the other groups. In the injection study and the dose response study the DTH assay showed hypersensitivity of the sensitised T-cells and suppression of antibody response in the exposed groups. A past study examining the effect of DDT on the DTH reaction to bovine serum albumin found that exposure elevated the response [119]. Recent studies that have applied this technique to mammalian and avian systems, have found the opposite scenario, with exposed regions causing a suppression of this response. These studies were mainly dealing with industrial contaminants rather than pesticides, however, and the immunological effects might be quite different. Another study, which focused on wild birds, looked at species that were exposed to pesticides and found stimulatory rather than suppressive effects. Tree swallow chicks reared in pesticide sprayed orchards showed significantly stimulated T- and B-cell blastogenic response and a reduction in thymic maturation which correlated with increasing pesticides. Contaminant analysis of the eggs revealed *p,p'*-DDE, was as high as 2.29 μg/g wet weight and was the only elevated compound. The frequency of the pesticide exposure in these birds was correlated with the immunostimulatory effects found [87]. The results of contamination may not be suppression of the immune system, some endpoints can be stimulated. The consequences of immune stimulation due to pesticide exposure may be as detrimental as

a decline in immune system function [87]. For instance autoimmunity and hypersensitivity are possible outcomes of stimulated immune system function [87].

3.4.3 Chemiluminescence

The initial CL assay also showed differences between the groups. The responses of the frogs in Collingwood, Ottawa and Pelee groups were all elevated in comparison to the other groups. This elevation of Ottawa and Collingwood groups, considered to come from cleaner areas of Ontario, could be considered to show suppression of the responses of the other groups if it were not for the high value of the Point Pelee frogs at this time point. When the assay was repeated two months later the peak CL of all groups except Holiday Beach and Pelee were higher. This increase was most dramatic in the Holiday Beach group, but this was particularly attributable to the response of one frog rather than the group as a whole. When the results were plotted these high values were omitted as they were termed outliers by the studentized residual test generated by Systat.

The results of this assay appeared to be quite variable and there are a few factors which can greatly affect the CL assay results. There is a time component which can greatly affect the success and accuracy of the CL results. For example it is necessary for the blood to be processed within 10 hours of being drawn. During the optimization of this assay by Marnila et al [58], it was found that the CL response increased 1±4% in the first 2 hours, although the peak value did not change. The intensities of phagocyte CL emissions were also found to be dependant upon temperature. When two plates were to be run consecutively it was normal practice to put the second plate, wrapped in tin foil into the fridge. The peak CL of the second group might then be very much lower than

the first group not due to differences in the respiratory burst activities of the cells but due to the effect of temperature.

3.4.5 Experimental Considerations

The importance of undertaking the assays as quickly as possible after the frogs have been removed from the field are outlined by the differences between the results of the initial immune assays and those obtained two months later. Variation in the responses of the various groups might in part have been due to the stress of travel and confinement. Stress can alter immune function and make animals more susceptible to infection. Increased plasma corticosterone levels have been shown to cause definite alterations in immune functions [89]. Corticosteroids are known to exert a variety of additional effects on immune activities in ectotherms. They can have both an anti-inflammatory or inflammatory immune responses depending upon the concentration, physical state of the animal, timing of exposure and the immune assay being tested [91]. Field collection at various sites take time and this meant some frogs were resident at GLIER for longer than others before the immune studies were initiated. In addition, the frogs collected from the Ottawa and Collingwood regions were under intense stress for a much longer period of time than the Essex county frogs who were captured and placed in aquaria the same night that they were captured. The frogs from Ottawa were confined in transit for the greatest length of time (four to five days), followed by those from Collingwood (two to three days).

A study design which did not remove the animals from their home range might be more appropriate to the accurate measurement of immune function in frogs from various areas. For example if a caged study at the site was conducted the problem of transport

and captive stress for the frogs might be alleviated. In addition the contaminant exposure of the individuals would also remain constant. The logistics of a study of this kind might be difficult in light of the availability of instrumentation and the lack of stability of the samples for the CL assay. The antibody response assay and the DTH would be applicable under these conditions as the plasma samples for the first assay can be frozen and the second assay only requires measurements with accurate callipers.

3.4.6 CONCLUSIONS

Spatial differences in various aspects of immune function were measured in frogs from different regions of Ontario. Although differences in contaminant burdens were apparent in the tissue of frogs these cannot conclusively be linked to the altered immune function between the sites. The altered antibody response, however, seemed to be reflective of the contaminant burdens for the Point Pelee group. In light of the fact that too few samples were taken for contaminant analysis and that mitigating factors such as stress might have altered the results, contaminants cannot be shown to be responsible for the changes. However the immunological assays developed are sensitive enough to measure differences between populations and there is great applicability for methods in assessing amphibian population health. Future studies which reduce stress and environmental alterations by assaying frogs maintained in a captive environment at their home site would give more insight into spatial differences between immune function in Ontario ranids. Further, development and optimisation of further assays should be conducted to gain a more reliable picture of the effects of pesticides on amphibian immune function.

CHAPTER 4.0 – GENERAL CONCLUSIONS

The results of the studies presented in this thesis suggest that exposure to pesticides can adversely impact upon the immune system of *Rana pipiens*. This was the first study to address immunological endpoints with relation to contaminant exposure in amphibians. It used non-sacrificial techniques and adaptations of published mammalian and avian assays to characterise immune alterations in amphibians. In addition wild collected frogs were successfully maintained under experimental laboratory conditions and periodically had blood samples taken using cardiac puncture without apparent ill effect.

The three assays which were used in these studies, the antibody response, CL and DTH, provided a useful starting point with which to base further studies. The most reliable of the three was the antibody response. This assay would be even more useful with the production of a *Rana* specific antibody because the binding of the *Xenopus* antibody that was used was likely not responsive to all Ontario ranids, and was weak even for the leopard frog. The DTH assay proved to be useful in that a similar trend of enhancement of T-cells was always apparent, although never significant. The complexities of making very small measurements such as toe width generated the error that was seen. This assay could be improved by refinement of the measuring instrument, site of injection and method. The CL assay provided results of innate immunity which changed over time. These changes appeared to mirror alterations in the humoral endpoint of antibody response. The results are, at times variable, as innate endpoints often are, but this variability was outweighed by the very small amount of sample required (2 μl of

whole blood) and the procedural ease of both sampling and performing the assay. Future studies should include the development of more immunological assays such as the lymphocyte proliferation to determine mitogen stimulation of T and B-cells and the use of the antibody AM 20 to determine cell surface expression of class II MHC (see initial development, appendix B).

In chapter two it was shown that alterations in immune function, following exposure to a sublethal dose of DDT, dieldrin and malathion, could be determined using these methods. The high concentrations of all three pesticides tested elicited suppressive and stimulatory effects in the selected endpoints. The pesticides used in this study, with the exception of malathion are no longer in use in North America. They were chosen because they are still present in some environments where frogs live and have previously been known to cause alterations in immune response in other species. During the twenty weeks over which the injection study experiment and the ten weeks for the dose response experiment were undertaken, the immune system of the frogs appeared to recover. This recovery did not, however, correlate to the clearance of OCs from the frogs system as shown by contaminant analysis. The reverse immunisation experiment established that if frogs came into contact with an immunological challenge such as a pathogen, before exposure to a pesticide, the impairment of their immune function would likely not occur. The antibody response assay proved to be the most reliable and informative of the three assays used. However, very low reactivity was occasionally experienced with the 6-16 antibody. This antibody was developed to mark *Xenopus* IgM rather than *Rana* IgM. In the future, the ELISA would likely be improved by the development of an antibody specific for *R. pipiens* IgM. The immunological parameters examined revealed the

importance of using assays that examine a variety of immune system components. One assay on its own might not reveal all changes in immune function. For example, during the dose response study, following DDT exposure, the antibody response to KLH was impaired but the CL response was stimulated. The increase in CL could be explained as a compensatory mechanism of the innate system by the frogs to the lowered antibody response.

Injected dosing of pesticides, though informative with regard to physiological changes, does not necessarily reflect either environmental route or dose of exposure in wild populations. A more accurate picture might emerge if immune endpoints were assayed at a variety of time points, during a feeding study which used very small quantities of pesticides in a food source, over an extended period of time.

In chapter three measurable differences in immune function were found to exist between the frogs collected from different regions of Ontario. In some instances these effects were stimulatory and in others they were suppressive. Very high levels of contaminants were found in one frog from Point Pelee. Although the contaminant results appeared to support the idea that immunological changes in wild caught populations were reflecting elevated environmental exposure, too few individuals were collected to make conclusive statements regarding the immune function to be made. It seems plausible, however, that pesticides will impact upon wild amphibian populations in the same way as they have altered parameters of laboratory maintained populations.

Two possible sources of inaccuracies in this study were identified. The first was associated with the methodology of the CL assay. The other was the increased stress that the frogs from Ottawa and Collingwood had to endure due to extended travel time.

The Collingwood frogs were not confined for as long a period as the Ottawa frogs. It is possible that the immune system of the Ottawa frogs might have differed from that of Collingwood as a result of travel stress.

This investigation shows that frogs could be considered good indicators of the effects of contaminants. Sparling [38] outlines the criteria necessary for selecting an organism as a bioindicator of contamination. The species should have at least moderate tolerance to the lethal or reproductive effects of the contaminants so that it is able to co-occur. The selected species should also occupy a trophic status or possess physiological characteristics to accumulate the compound at higher than ambient levels. The species should be static so that it is reflecting the area in which it is sampled and, in addition, it should be broadly distributed so that comparisons between regions can occur. The organism should be sufficiently common to allow harvesting and manipulation without concern for population survival. In order to measure residues or physiological changes the species should be of sufficient size [38]. Amphibians as a group meet all of these criteria and make suitable candidates as biomonitors and bioindicators of the accumulation and effects of many organic contaminants.

The importance of rapid analysis and stress reduction in obtaining an accurate immunological picture of an amphibian population was highlighted during the field study. Future field based investigations should concentrate on reducing the need to remove animals from the site. For example an *in situ* caged study where individuals are captured, identified by photographs, immunised and then maintained in a secure enclosure for two weeks to allow for antibody production, would reduce these stresses. In addition, seasonal changes such as differences in immune response during the spring

or fall and temperature associated immune changes should be determined to more completely understand what alterations are attributable to pesticide exposure.

This project examined the effects of pesticides on adult frogs collected from various regions and it established that significant differences in immunological parameters existed between groups of individuals from the different sites. The difficulty with this, however, was that the frogs used in the investigation are the surviving adults, natural selection has already eliminated those that suffered from drastically impaired immunity or other contaminant related effects such as loss of flight response. The use of adults was an obvious starting point for this project because their larger size allowed for the development of non-destructive sampling techniques and they had a fully developed immune system, which the tadpole stages lack. However, this approach did not account for the many effects that might be occurring in egg masses or tadpoles. Future studies should address all life stages and examine the trans-generational effects of adult contaminant burden.

Further studies examining immunological parameters of amphibians with respect to contaminant exposure would benefit by expanding the number and types of contaminants examined. Synergistic effects of a wide range of xenobiotics with each other should also be examined, along with the consequence of exposure in conjunction with ultraviolet-B (UV-B) rays [2]. In addition, the mode of action of different contaminants on the amphibian immunity should also be addressed to determine what component of the system each class of contaminant effects. This will become particularly important when linking pesticides or industrial chemicals to the incidence of disease in affected populations.

Although immunological studies are of great importance when considering contaminant effects, a study of this kind would be further strengthened by the incorporation of other physiological endpoints. For example there has recently been great interest in the alteration of endocrine related endpoints in other wildlife species and amphibians [12, 88]. This has been found to be a sensitive indicator of contaminant related stresses. In addition very subtle genotoxic effects have already been detected in pesticide exposed frogs in Quebec [120]. A future study which addressed immune changes in conjunction with endocrine, genotoxic and morphological abnormalities might produce a strengthened case regarding the role of chemicals in the decline of global amphibian populations.

The importance of extraneous factors such as stress, husbandry, capture time, assay inaccuracies was highlighted from the results of these investigations. Future studies in this area should be aimed at more positively identifying the impact of pesticides, on immune alterations in an *in situ* field setting. This will allow for a more comprehensive evaluation of the toxicological stress on anuran populations resulting from pesticide exposure and help to determine whether the use of pesticides is accelerating the decline of some frog species.

Overall this thesis has established that exposure to some pesticides does alter the immune function of leopard frogs. Differences in immune function in frogs from different regions of Ontario could be measured. Although definitive statements regarding the impact of elevated OC concentrations on immune function cannot be made strong associations appear to be present. Application of the further studies outlined above and repetition of the techniques investigated here will address the hypothesis that pesticides are altering

the immune capabilities of amphibians. Further, incorporation of endocrine, genotoxic and physiological endpoints with the existing immunological parameters, will reveal a more complete picture of the impacts that anthropogenic chemicals are having on amphibian populations worldwide.

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Appendix A - Pesticide Residues in Amphibians (ug/g)

Species	Age	Site	pp-DDT	pp-DDE	pp-DDD	dieldrin	Publication	Comments
Green Frog	Adult	Summerstown	5.7	26			Gillan, 1998	Field samples collected from various sites in S Ontario
Green Frog	Adult	Cornwall	ND	ND				
Green Frog	Adult	Brighton	22.6	174				
Green Frog	Adult	Ancaster	16	754				
Green Frog	Adult	Longpoint	59	252				
Green Frog	Adult	Rondeau	48	179				
Green Frog	Adult	Ojibway	27	156				
Green Frog	Adult	Hilman Marsh	60	284				
Leopard Frog	Adult	Cornwall	ND	83				
Leopard Frog	Adult	Kingston	ND	60				
Leopard Frog	Adult	Brighton	ND	232				
Leopard Frog	Adult	Ancaster	80	572				
Leopard Frog	Adult	Longpoint	9.6	659				
Leopard Frog	Adult	Rondeau	48	179				
Leopard Frog	Adult	Ojibway	18	125				
Spring Peeper	Adult	Point Pelee	160.6	1001.1	26.4	199.8	Russell, 1995	as above
Green Frog	Adult	Summerstown		0.58			Russell, 1997	as above
Green Frog	Adult	Brighton		2.17				
Green Frog	Adult	Ancaster		45.02				
Green Frog	Adult	Longpoint		3.54				
Green Frog	Adult	Rondeau		1.5				
Green Frog	Adult	Hilman Marsh		5.84				
Green Frog	Adult	Ojibway		5.45				

Appendix A - Continued

Species	Age	Site	pp-DDT	pp-DDE	pp-DDD	dieldrin	Publication	Comments
Spotted Frog	Adult	Oregon (live)	563-1750	96-173	166-346		Kirk, 1988	DDT/ha three weeks after spraying in 1973, some were live on capture and others were dead frogs found at the site
Spotted Frog	Adult	Oregon (dead)	122-5670	96-366	1920-6670			
Mudpuppy	Adult	Ottawa R	0.1-13.8	41.5-488	4.9-98.4	3.4-16.6	Gendron, 1997	Field collected
Mudpuppy	Adult	St. Law. R.	0.5-8.3	0.3-90.0	1.7-24.8	>DL-25.7	Bonin, 1997	Field collected
Leopard Frog	Adult	Lake Erie	30				Meeks, 1968	Exposed to 36Cl-DDT and measured 1 year later
Bullfrog	Adult	Lake Erie	400					
American Toad	Adult	Montana	100			1400	Korschgen 1970	Collected from cornfields in 1967
American Toad	Juvenile	Montana	100			4600		

APPENDIX B
METHOD DEVELOPMENT AND OPTIMIZATION

Numerous techniques were examined, developed and optimised during this project. These included methods to capture and successfully care for the experimental animals as well as a variety of immunological assays. This section will detail the techniques that were established, or partially developed, to serve as a methodology record for future research in this area.

Much of the initial stages of this study dealt with the difficulties of maintaining wild caught frogs in a laboratory environment. Attempts were made to make the habitat which they were maintained as ideal as possible to minimise the adverse physiological impacts of stress. Disease within the population had obvious implications on the outcome of the experimental results and efforts were made to limit outbreaks through husbandry practices. This alleviated the need for treatment and the subsequent possible variation in the data obtained.

One limiting factor in these studies was the number of available animals. For the injection study, the reverse order immunisation study and the dose response study, animals which had been captured from a relatively clean area and maintained in captivity for approximately six months to a year were thought most suitable. These frogs were accustomed to captive living, some contaminants had likely cleared from their system and considerations such as diet, condition and hibernation period could be monitored. Individuals used for the method development stages of the study were not used again in any of the contaminant exposure experiments.

The immunological assays that were developed attempted to address both the innate and adaptive immunity. Assays that examine adaptive sections of the immune system such as immunoglobulin production, are often more informative and indicative of the true immunological function of the individual, than those that examine non-specific initial immune endpoints, such as phagocytosis. The nature of these assays however requires the production of species specific immunological markers such as antibodies, which are labour intensive to develop. For the purpose of this study antibodies that had been developed for use in other anuran species were kindly donated by other researchers and utilised.

The objective was to design assays which were non-sacrificial in nature so that eventual applications in long term field based studies, which did not further impact depleted populations, could be utilised. Difficulties arose due to the size of the species and the amount of blood that could be collected safely from each individual. Initially four or five assays were planned for each individual at one time but blood volume was found to be an impediment. Complications also arose due to reduced availability of some instrumentation (flow cytometer) at the University of Windsor that necessitated travel to the medical school in Detroit. This limited the use of some of the assays that were dependent upon these facilities in the light of travel and time restrictions.

A.1 Field Collection

Northern leopard frogs *(Rana pipiens)* were collected from streams, ponds, marshes, grasslands and meadows. They appeared to be most abundant in cultivated hay fields or meadows grazed by cows or horses. Collection was undertaken with a large, fine

mesh, long handled trout net. The technique involved walking slowly along a suitable site until the movement disturbed a frog. The net was then dropped onto the area where the frog had jumped and held down. The frog could then be located within the confines of the net area and caught from above by hand. During collection the frogs were placed into small plastic carrying containers with fliptop lids. Periodically this container was emptied into a large, communal container filled with a several inches of water so that the frogs were able to float, but could not jump. Steps were taken to ensure frogs did not overheat, for example the vehicle was parked in shaded areas and ice was added if necessary. While in the field, handling was kept to a minimum to reduce stress and minimise the possibility of escape.

A.2 Species Description

Leopard frogs grow to about five inches in length. They are slender, long-legged frogs with two conspicuous dorsolateral ridges. They are marked on the back, sides and legs with numerous dark, round spots that usually have light boarders around them. They are usually green, although some may be light brown in colour. The call is sometimes compared to the sound of wet hands rubbing across a wet balloon.

Both sexes look similar, though males are generally smaller than females, reaching only three and one quarter inches. Males have two vocal sacs that swell out from their armpits when the frogs are calling, and during the breeding season, the thumbs on the males' front feet swell in size. Females usually grow to three and a half inches or more in length. During the breeding season they are much fatter than males.

A.3 Laboratory Care

Upon arrival, each frog was allocated an identification sheet, which covered such aspects as colour, species, sex, length and weight. This served as a record to monitor weight changes, growth and experimental history. For identification purposes each frog was photographed , the spot pattern was drawn into a frog shaped outline on the sheet and a description was written for each individual . In addition, daily information was recorded on frog care sheets which dealt with issues such as daily cleaning, feeding, mortality and escapes.

The frogs were maintained in the aquatic facility at the Great Lakes Institute for Environmental Research (GLIER). They were housed in aquariums (30 x 90 cm) in groups of five and provided with a washed concrete block covered in rubber matting as a cricket feeding platform. The aquariums had purpose built wooden lids, with a plastic mesh interior, which fit tightly making escapes difficult. Underwater charcoal filters (Quick~Filter® (802), Hagen) were used to remove waste products from the water and the aquaria were cleaned every second day by siphoning the waste and dirt, and then refilling with chlorinated water. The frogs were left in the tank during the procedure. Latex lab gloves were worn at all times whilst handling the frogs to minimise their exposure to foreign compounds. When handled the frogs were held around their waist region and efforts were made to make the experience as quick and as stress free as possible.

The frogs were maintained on a diet of crickets (Pet Depot, Windsor, Ontario), which were kept in large glass aquariums and maintained on a diet bird granules

(Tropican, Hagen, Inc, Montreal). The crickets were placed on top of rubber covered concrete blocks when fed to the frogs.

A.4 Disease Outbreaks

The main disease problems experienced were periodic outbreaks of red-leg disease. This is a non-specific term for a variety of symptoms, caused by exposures to a range of bacterial of factors such as *Aeromonas hydrophila, Bacterium alkaligenes, Mimeae* and *Staphylococcus epidermis*. Without culturing samples from the water or the animal it is impossible to know which agent is causing the symptoms [121]. The symptoms displayed included loss of appetite, weight loss, dilated pupils, lacking brilliance in skin coloration, slumped posture, disinclination to move, tense abdomen, cutaneous haemorrhaging, eroded feet, toes or jaws. The majority of deaths in laboratory frogs are caused by bacteria and viruses and stress is known to be a major factor in disease outbreaks [16, 121]. Bacterial diseases can seriously affect experimental results, especially as immunological endpoints are being assessed, so prevention is vital. There are a number of treatment methods that can be utilised, though care was taken not to treat any frogs throughout the duration of the experiments for fear of altering the results. If an animal became too ill to be humanly used for the experiment it was euthanized.

In order to prevent bacterial disease from occurring the most important factors recognised were stress reduction, cleanliness, reduced numbers of individuals in each tank and an appropriate male:female ratio. Wild caught frogs were found to be more difficult to keep than frogs that were acclimatised to the laboratory conditions. It is probable that this is due to the stress of capture and containment. A few factors were

found to be important in reducing the onset of these symptoms in wild caught frogs. Stress reduction was deemed the vital factor. This was achieved by designing the aquarium environment to have "caves" constructed of plastic containers, so the frogs were able to hide out of sight. Handling stress was reduced by minimising the need to pick the animals up, reducing the procedure time and wrapping the frog in wet paper towels during bleeding procedures to keep them in a moist, dark area. Maintaining the frogs in small groups, of five to six animals also reduced stress. The number of males within the group was limited to two as this reduced fighting and territory disputes. Excess food was provided on the allocated feeding days, also to reduce confrontation. Frequent cleaning, carbon filtration and the use of chlorinated water limited the bacterial loading of the water. Non-hibernating *R. pipiens* adults can tolerate between 4 and 6 mg/L of chlorine [121], and its presence in the water will retard bacteria growth. Throughout the duration of this project red-leg symptoms were only experienced when the frogs were maintained in non-chlorinated water and receded shortly after tap water replaced the treated dechlorinated supply.

A.4.1 Treatment

Treatment of bacterial diseases was undertaken for individuals that were not part of an experiment. The animal should be immediately isolated to stop the spread to other individuals, and the quarters should be thoroughly cleaned using Wescodyne (Amsco (Steris) International Inc, Menter, Ohio). Soluble tetracycline (25mg/mL in deionized water) was found to be the best antibiotic on the basis of sensitivity and wide tissue distribution [121]. An oral dose of 5mg/ 30 g of frog, administered through a stomach

tube, twice a day for 7 days was found to be the best method. Putting amounts of tetracycline in the tank water serves no useful purpose, as not enough enters frog and it causes skin lesions [121]. Injections are also not an appropriate route as they cause a localised reaction. Throughout the treatment period it is important to keep the water clean as excreted tetracycline can cause burning of the skin. The treatment also causes nausea, so food should not be offered, as vomiting can follow and then the treatment is lost.

A.5 Immunological Assays

A.5.1 Blood Collection

Blood was collected by cardiac puncture in a method outlined by Dr. J. Sweetman (unpublished), a local veterinarian who specialises in reptile and amphibian medicine. Initially the amount of blood that could safely be removed from each frog was calculated by assuming that 6% of the weight of the frog is composed of blood and 10% of this blood can be utilised. With experience, it became apparent that on average leopard frogs weighing between 20 and 50 grams, could yield a sample of 0.4 – 0.7 mL of blood, every two weeks, without apparent ill effect.

To restrain the frog during the procedure, it was wrapped snugly in moist paper towel ensuring that the legs were straight out. A small area in the paper towel was opened at the injection site. A sterile, disposable 1 ml syringe with a removable 28G x ½ needle coated with the anticoagulantant, lithium heparin, were used to collect the sample. The needle was inserted at a 45° angle into the bottom of the sternum, and slight pressure applied on the plunger until the blood started to flow, the heart beat allowed the syringe

to fill. Once the amount of blood required was obtained, the needle was gently removed and the blood aliquoted slowly into an Eppendorf tube. Almost immediately after being removed from the paper towel restraint, the frogs recovered. Throughout the duration of this project no mortality, which could be directly attributable to the cardiac puncture procedure, were observed.

A.5.2 Enzyme Linked Immunosorbent Assay

The method used for the ELISA is outlined in chapter 2, section 2.2.5, page26. The developmental stages of the assay are presented here. Two separate antibody types were kindly donated and the binding in Ontario ranids was investigated by ELISA. The first type was 38-1 and 38-2, rabbit anti *Rana catesbiana* high molecular weight immunoglobulins provided by L. Steiner [122, 123]. The second type was 6-16, a monoclonal antibody, donated by M. Flajnik. This antibody has been found to react with the IgM of some *Xenopus* species [124] and cross reacts to some extent with *Rana* species.

To ascertain the binding of the antibodies, an ELISA was undertaken to determine whether the 6-16 would bind to non-specific IgM antibodies in leopard frog plasma and whether 38-1 and 38-2 antibodies would indicate the presence of non-specific high molecular weight Igs. The method used was a slightly modified version of the protocol set out in chapter 2, section 2.2.5. The KLH-DNP protein was not pre-injected into the frogs. The ELISA plates were coated with 100 µl leopard frog plasma diluted 1:2 in coating buffer (15mM Na_2CO_3, 34.88mM $NaHCO_3$, 3.125mM NaN_3 pH 9.6). Figure A1 shows the binding of 6-16 antibody to total IgM in leopard frog plasma. The antibodies

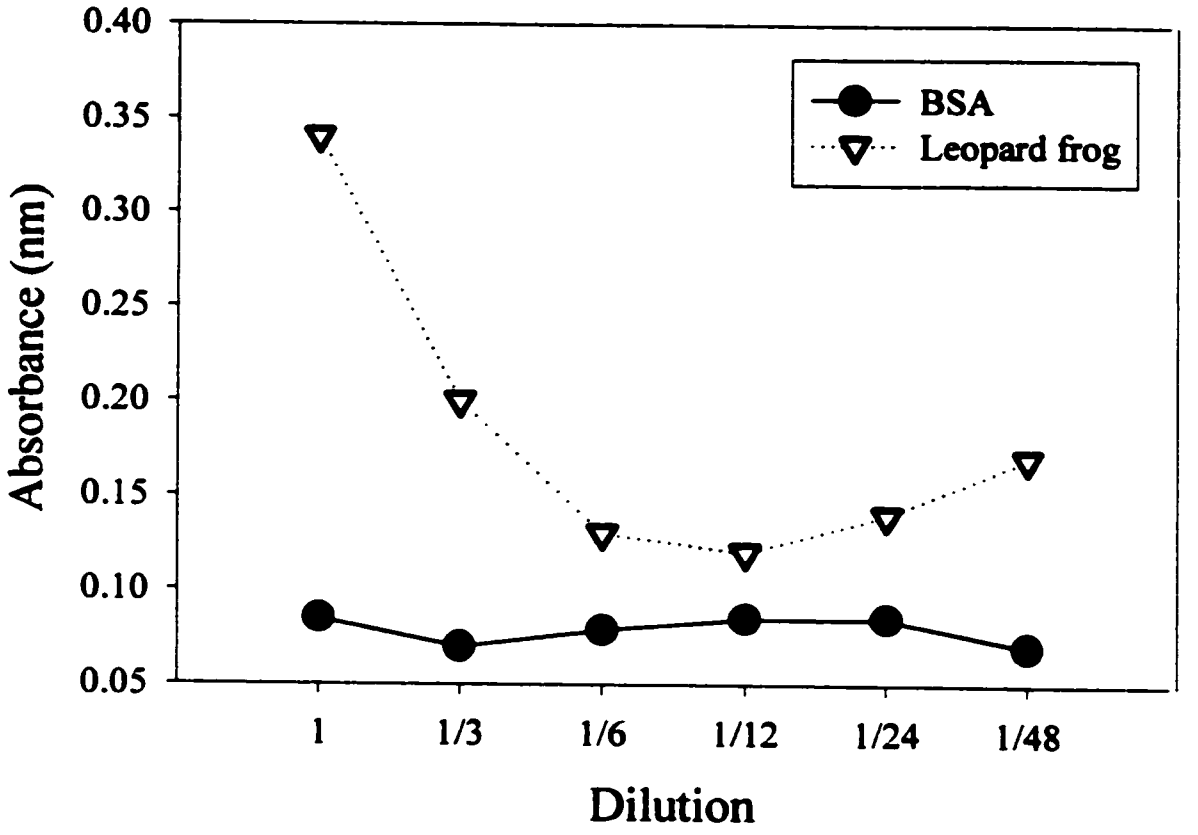

FIGURE A1 – Reactivity of leopard frog serum to anti-*Xenopus* IgM 6-16, n=4.

38-1 and 38-2 were also found to react with Ig in *Rana* plasma producing a measurable colour change. For the final ELISA 6-16 was used due to its availability and specificity to IgM.

The capacity of different blocking buffers to stop non-specific plate binding was examined. Initially bovine serum albumin (BSA) was used as the blocking solution, but was found to be inappropriate as some reaction with the secondary antibody occurred possibly due to the similarity in molecular weight with the immunoglobulin. The other blocking buffers used were 5% gelatin, blotto and blotto-tween. The blotto was made up of 5% Carnation instant milk powder in PBS and the blotto-tween had 0.2 % tween also added. The gelatin was found to be the best blocking solution as all others showed some binding in the control wells. Initially 100 µl was added to the wells, but non-specific binding occurred as this was the same volume as the plasma and the antibodies aliquots. By adding 250 µl and incubating for 30 minutes, this binding was alleviated.

A.5.3 Chemluminescence

The chemiluminescence assay was developed by Marnila *et al.*, [58] for *Rana sp.*, and was undertaken in exactly the manner described and no further development was necessary.

Two separate luminometers were used, for the injection study a MLX Microtiter® Plate Luminometer system was used and for the dose response a Wallac 1450 Microbeta Trilux with a 2" detector was used. The two systems worked on different output systems of relative light units (Windsor) and counts per minute (Waterloo). This means that the

numbers obtained between the dose response and the injection study are not analogous. The trends obtained can be compared, but actual numbers cannot.

A.5.4 Delayed –Type Hypersensitivity

The procedure for the delayed type hypersensitivity (DTH) is described in chapter 2, section 2.2.6. In the eventual assay PHA-P was used as the immunogenic compound to stimulate a reaction. This compound was decided upon after an initial investigation examining KLH, PHA-P and ovalbumin, to determine the best compounds to use to obtain a measurable DTH reaction. Other factors were also investigated such as measuring site, reaction time, calliper type, and human measuring error.

A.5.4.1 Measurement Site

Due to the small size of a leopard frog, difficulties arose when deciding upon a suitable reaction site to measure. One study which used DTH as a tool to quantify the effect of industrial contaminants on the immune system of seals, used the toe web as the injection site [125]. Another study that used PHA-P as the reactive agent in a skin scratch reaction in fish-eating birds, used the wing web as the measuring site [93]. Initially the toe web of the frog was thought to be a suitable measuring site. This was found to be erroneous however as the frog toe web was too variable to allow repeated sampling of the same site, too thin to ensure that the injected compound was retained and the thickness varied greatly with the tension applied by the handler. Eventually the width of the middle toe was selected as a site to provide a more repeatable value. Measurements occurred at the point where the webbing on either side of the middle toe

meet the digit, so that duplicates were as accurate as possible. In an attempt to increase accuracy, injections of the immunogenic compound occurred on the same side of the toe each time and were administered at the measuring site. To reduce operator variation, one person was responsible for all measurements throughout the duration of the experiment.

A.5.4.2 Investigation of Immunogenic Compounds

Solutions of 2 mg/mL of KLH, PHA-P and ovalbumin were made up and frogs were pre-immunized with 50 µl of these immunogenic compounds. After nine days initial calliper readings of the left and right toe were taken. The left toe was then injected with 10 µl of the immunogenic solution. As a control for inflammation caused by the injection, the right toe was injected with 10 µl of PBS.

The results of this experiment are shown in Figure A2. Ovalbumin did not produce a measurable response. The response of PHA-P was the highest, followed by KLH. Following these results PHA-P was selected as the immunogenic compound of choice for the DTH experiments.

A.5.4.3 PHA-P ELISA

To determine whether the measured reaction of frogs to PHA-P was a DTH reaction or a skin scratch, an ELISA was performed on the plasma of frogs immunised 2 weeks previously. In exactly the same manner as the KLH ELISA, 96 well plates were coated overnight with 2 mg/mL PHA in coating buffer. The results are shown in Figure A3. PHA-P is a mitogen so the results do not correlate with the protein antibody alterations in

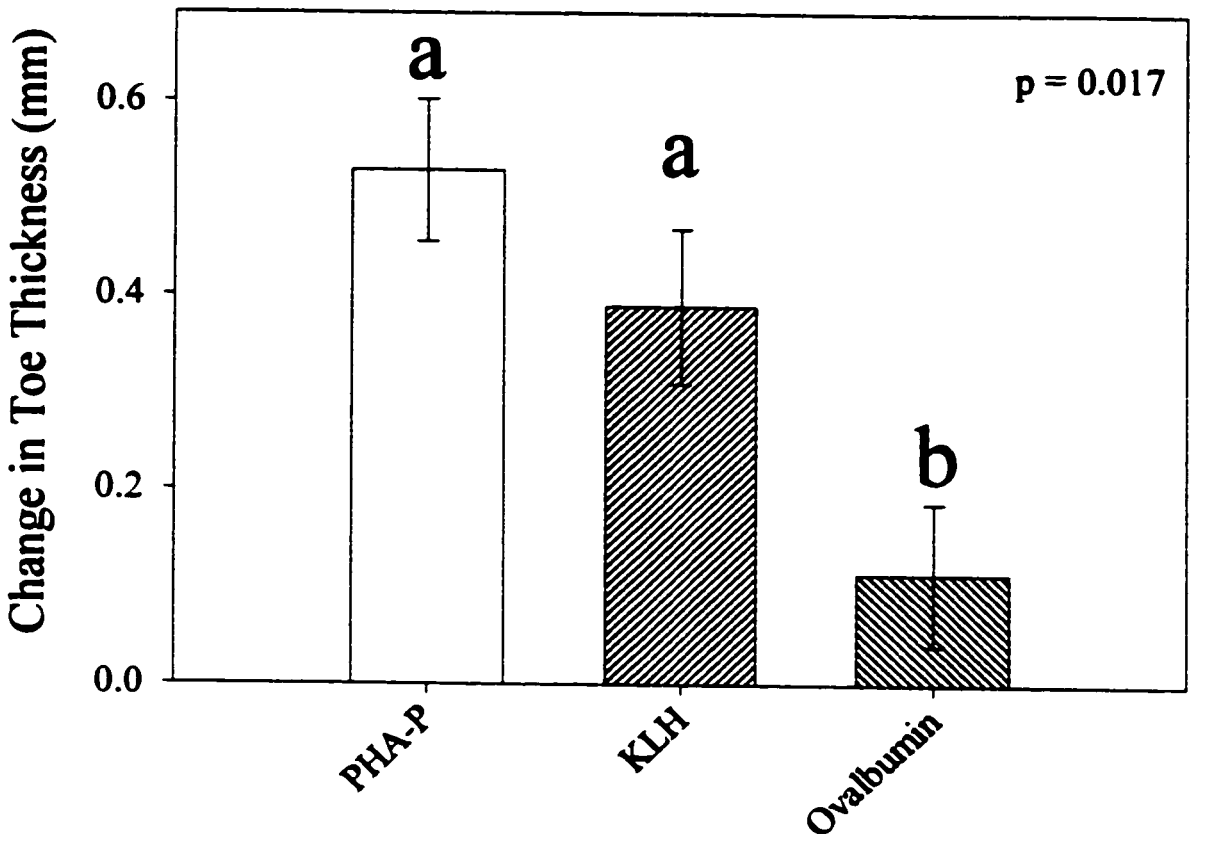

Figure A2 – A comparison of the delayed type hypersensitivity reaction as measured by an increase in toe thickness in response to PHA-P, KLH and ovalbumin. Data are presented as the mean ± standard error, n = 4.

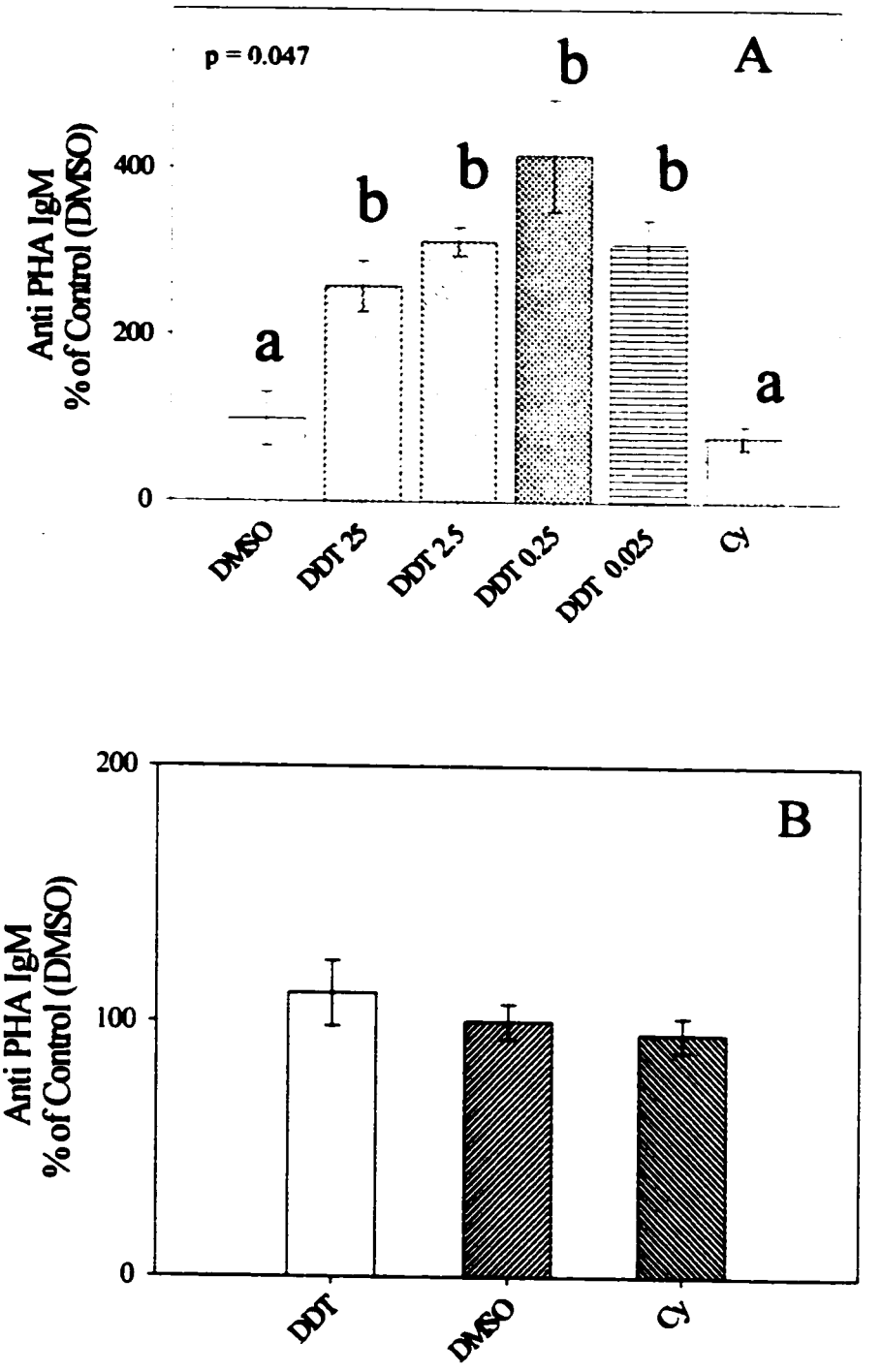

Figure A3 - Anti PHA IgM levels measured by ELISA two weeks after contaminant exposure in A) dose response and B) reverse order immunization studies. For dose response n = 5 and for reverse immunization n=4. Differences were shown by one way ANOVA using bonferroni adjustment to generate p values.

response to KLH-DNP, but they do show that antibodies to PHA-P exist in the plasma of preimmunised frogs. An immunological memory is therefore in place. This indicates that the localised toe swelling in response to a PHA-P injection is indicative of sensitised T-cell infiltration.

A.5.4.4 DTH Measurement Error

To determine the measurement error that occurred in the interpretation of the calliper reading, a blind study was conducted. The right and left toe web of four frogs, were repeatedly measured in a random order, unknown to the measurer until five measurements for each frog were obtained. After five random readings the average standard deviation from the mean was found to be 0.166.

A.5.5 Cell Surface Expression of Class II MHC

A method for isolating lymphocytes from whole blood and marking them with AM20 is outlined here. AM20, is a monoclonal antibody which is reactive with *Xenopus* T-cells [126], and has been used to examine the cell surface expression of class II major histocompatability (MHC) on isolated frog lymphocytes. The stages outlined include materials and methods for the isolation and marking of lymphocytes using AM20, confirmation of the binding of AM20 and 6-16, the antibody used in the ELISA assay using confocal microscopy and the application of this method during the injection study.

A.5.5.1 Materials and Method

Fresh blood was centrifuged for 2 minutes at 600 x G, the plasma was removed and retained for further analysis (anti-KLH ELISA and contaminant determination). The remaining sample was rediluted to 3 times its original volume with phosphate buffered saline (PBS). The peripheral blood leukocytes (PBL) were isolated over a histopaque gradient (1077, Sigma Diagnostics, Inc., St Louis, MO). The diluted blood was layered over 3 mL of histopaque at room temperature. The samples were centrifuged at 400 x g for 30 minutes and the cells harvested from the interface. The samples were then washed once in 10 mL of PBS, mixed gently, centrifuged at 250 x g for 10 minutes and resuspended in 0.5 mL PBS. The PBL were incubated for 60 minutes on ice in 0.5 mLs of neat AM 20 (provided by M Flajnik). After incubation the cells were washed by centrifuging at 600 x G for 2 minute, they were then rinsed with tris buffered saline (TBS-T). The binding of the AM20 antibody was detected by incubating the cells for 30 minutes on ice with fluorescein isothiocyanate (FITC) conjugated goat anti-mouse immunoglobulin antibody diluted 1 in 100 TBS-T. The samples were washed twice in TBS-T and then preserved in 70% ethanol and analysed by flow cytometry at 488 nm at Wayne State University.

A.5.5.2 Confocal Laser Scanning Microscopy Analysis of 6-16 and AM20

Confirmation that antibody binding of AM20, a monoclonal antibody which is an IgG2a isotype and 16-6 which binds to IgM and is IgG1 isotype, was validated by using fluorescent tags to identify the cells and confocal laser scanning microscopy (CLSM) was used to view the fluorescence.

A.5.5.2.1 Experimental Design

The blood of six leopard frogs was collected by cardiac puncture using a heparinized 1 ml syringe and a 28½ gauge needle. Blood was stored at 4°C until for a maximum of 2 hours. The PBL were isolated as outlined above in 2.5.5.1. The six samples were split into two groups of three and incubated with AM20 or 6-16. The cells were incubated in 0.5 mL of neat antibody for 60 minutes on ice. The binding of the antibodies was detected by incubating the cells for 30 minutes on ice with biotyntalated Texas Red for AM20 detection and FITC for 6-16. The remaining antibody was thoroughly washed and the images viewed and overlaid using the software, Confocal Assistant.

Two methods were used to view the cells, one was to look at live cells and the other a preserved sample. Live cells were viewed by cutting a small well into parafilm on the surface of the slide. A 20µl sample was then placed into the well and a cover slip put over the top. For the preserved sample, the cells were centrifuged, resuspended in ethanol and placed directly onto a microscope slide and covered with a coverslip. The cells were examined under both blue and green fluorescence using confocal microscopy and computer generated images using Confocal Assistant.

A.5.5.2.2 Results

The results show examples of two types of images that were generated. Figure A4a) shows a 6-16 lymphocyte which has been labelled by FITC, this indicates binding by the 6-16 antibody. Figure A4b) shows a lymphocyte labelled with Texas Red

and indicating binding of AM20 antibody. There was some difference using live cells in the parafilm wells as opposed to preserved cells. The live cells had a better shape but needed to be examined fairly quickly as the sample deteriorated with time and exposure to the laser.

The results show that both of the antibodies did bind onto cells present in the isolated lymphocyte fraction after histopaque purification. The AM20 antibody is marking class II MHC receptors which present peptide fragments of extracellular pathogens to helper T-cells [126]. These become activated and secrete factors that cause B-cells to initiate antibody production. MHC class II molecules are expressed on those cells capable of presenting antigen in order to stimulate an immune response.

The binding of the 6-16 was also apparent in *R. pipiens* cells, as the antibody was raised against *Xenopus* and designed to be specific for that species, this is surprising. It was determined to be specific for Xenopus IgM by functional and imunochemical criteria [108]. One study examining 6-16, found that it did react uniformly with most of the *Xenopus* species and subspecies tested [124] which suggests higher polymorphism than the paper indicates.

A.5.5.3 Cell Surface Expression of Class II MHC as Determined by Flow Cytometry in Pesticide Exposed Leopard Frogs

During the injection study isolated lymphocytes from twenty-five leopard frogs, were used to determine the differences in cell surface expression of class II MHC, 2 weeks and 4 weeks after exposure to DMSO, DDT, dieldrin, malathion and Cy. The

design and layout of this study are discussed in more detail in chapter 2. The isolation and preparation of lymphocytes was carried out as outlined in section A.5.5.1.

Flow cytometry is the characterisation of single cells as they pass at speeds up to 30,000 cells/second through a laser beam. Cells are characterised individually by quantifying fluorescence measurements. The laser acting as a light source develops parameters of light scatter as well as exciting the fluorescent molecules used to label the cell. Cells express antigens on their surface and immunoglobulins bind to cell surface antigen epitotpes very specifically. By tagging each antibody with a different coloured fluorochrome it is easy to distinguish the cell type and quantity of antigens expressed by each cell. A region can then be assigned which describes the amount of fluorescence being detected.

A.5.5.3.1 Results

The results of this assay when plotted as mean percentage gated in the M2 area, two weeks after contaminant exposure, can be seen in Figure A5. The M2 area was thought to be the region in which the fluorescent levels indicates phagocytes which had ingested three of more beads. There is statistically no difference between the groups 2 weeks after exposure although some differences are apparent at the 4 week point, when the contaminant groups appear statistically higher than the DMSO control group or the Cy frogs.

This may be a false result however, as when the shape of the flow cytometry peaks are compared between the two weeks a shift in the M1 area into the M2 area is

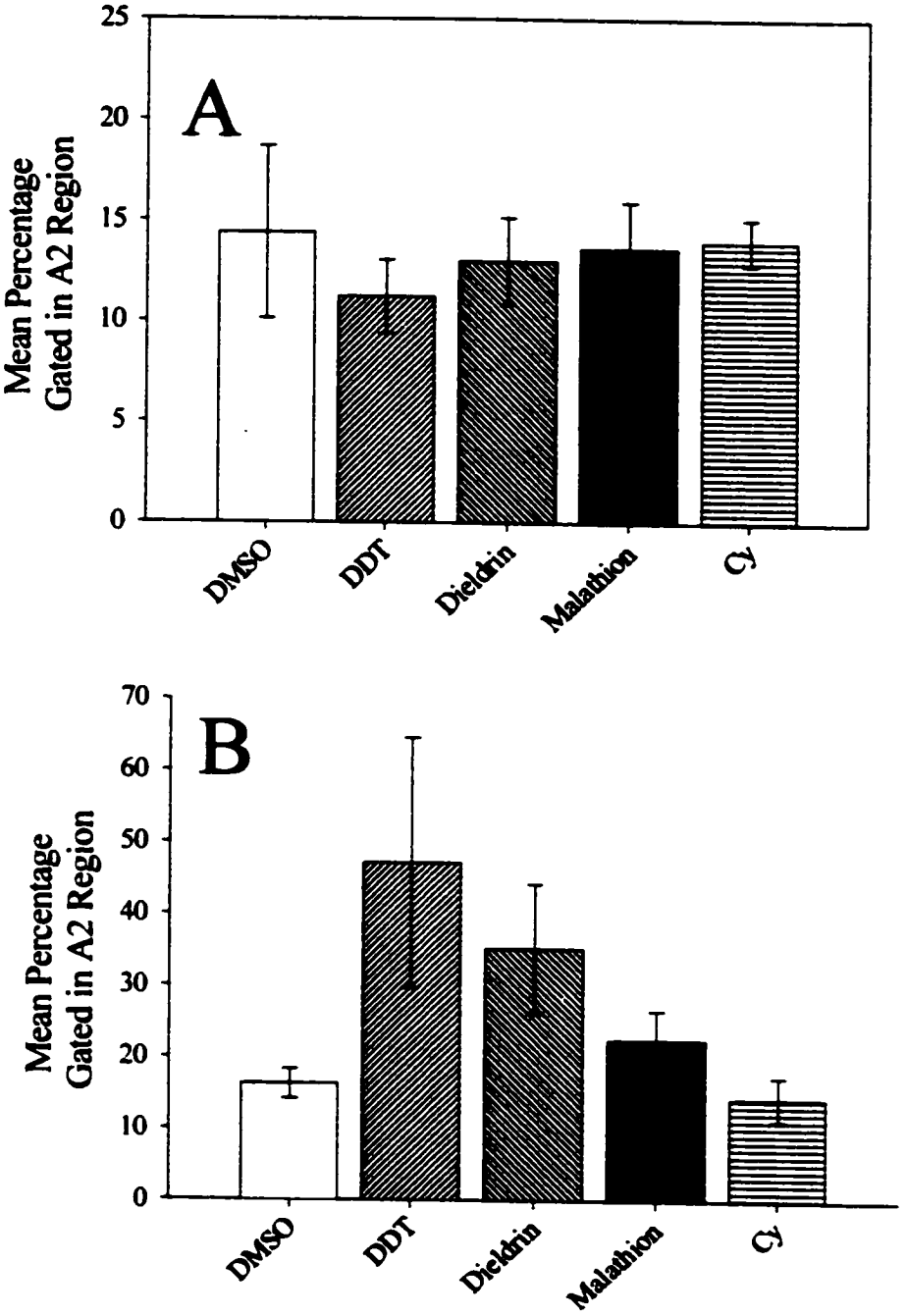

Figure A5 – Mean percentage of cells gated in the A2 region, which depicted binding of the AM20 antibody to the surface of lymphocytes A) two weeks after exposure and B) 4 weeks after exposure n=5 per group

apparent in some of the DDT and dieldrin exposed frog samples. This is shown in Figure A6 where, during the injection study, the shape of the peak at M2 region at A) 2 weeks and B) 4 weeks.

There are a few possible explanations for these differences. Firstly variation occurred in the number of cells in each sample. This might reflect losses during the washing stages or a variation in the amount of blood collected. These discrepancies could be eradicated by performing a cell count on each sample and ensuring that the preserved final sample is always rediluted to the same number of cells per mL. Another factor, which should be investigated, was the high value for non-specific binding. This might be reduced by the addition of a blocking agent, such as bovine serum albumin (BSA) between the AM20 binding stage and the FITC labelled secondary antibody. Care to wash the samples thoroughly, whilst not losing cell quantity, must also be accomplished.

In summary isolated lymphocytes can be obtained from small quantities of blood. The AM20 antibody is successfully binding to the cell surface of these lymphocytes, as shown by confocal microscopy. Flow cytometric analysis is an appropriate quantitative tool to measure this binding, but more efficient blocking is required to reduce non-specific binding.

A.5.6 Phagocytosis

Both of the assays described here for phagocytosis were adapted from a protocol kindly donated by Dr. P. Brousseasu, for use on Ontario ranid species.

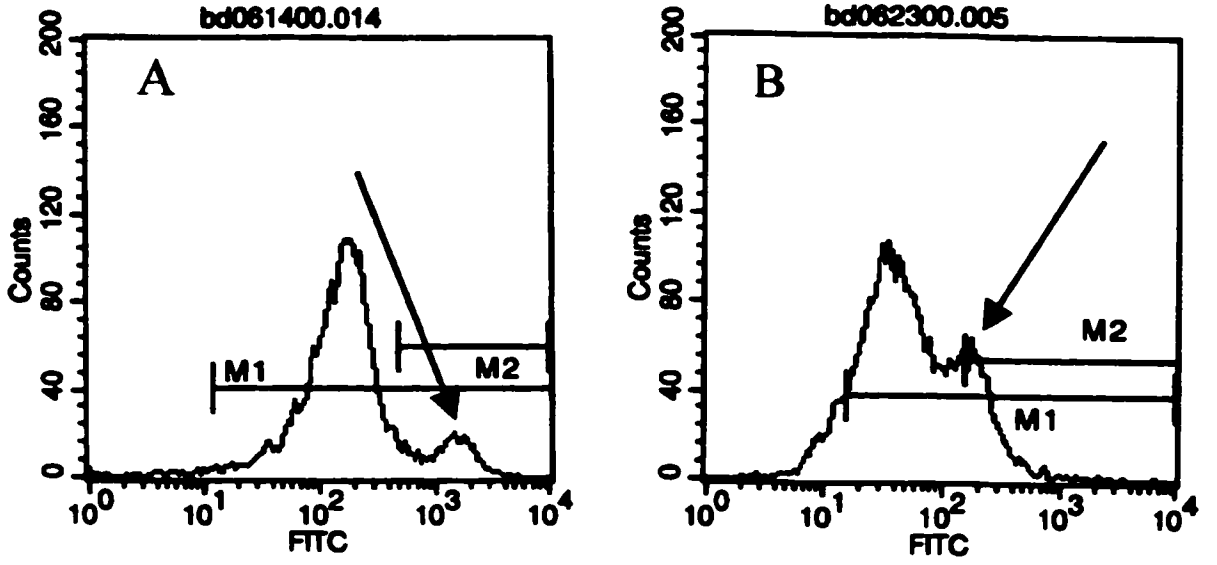

Figure A6 - An example of the shift in the M2 region in the injection study between A) 2 weeks and B) 4 weeks. The arrows denote the region which was thought to denote AM20 binding

A.5.6.1 Phagocytosis of Flourescent Microspheres of Spleen Cell Suspensions

The spleen was collected and a cell suspension prepared in L-15 supplemented with 10% FCS, 100 u penicillin, 100 µg/ml streptomycin by physically pushing the tissue though a stainless steel mesh. The suspension was washed once with the same medium by centrifuging 600 x G for 2 minutes, decanting the supernatant and adjusting the cell concentration was to 2 x 10^5 cells/mL. The cell suspension (0.5 ml) was distributed in a 12 x 75 mm polypropylene tube and flouresbrite carboxolate microspheres (1.716µ diameter, Polysciences Inc, Warrington, PA) were added at a ratio of 1:100 cell:beads. The sample was incubated in the dark, at 26°C under light agitation for 18 hours. Free beads were removed from the suspension over a 3% bovine serum albumin (Sigma Chemical Company) gradient. Acquisition was perform using a flow cytometer, 10,000 cells were analysed and results expressed as percentage of cells that phagocytosed 3 beads or more.

During the injection study (outlined page 65, section 3.3.2) frogs from dosed groups were sacrificed 8 weeks after exposure to various contaminants. Spleens were collected and the phagocytic assay undertaken as outlined above. The results are shown in Figure A7. There is statistically no difference between the groups as determined by a one way ANOVA. As this assay is a measure of phagocytic activity it was thought that it might correlate to the chemiluminescence assay. The results of this assay at the same time point are shown in section 3.4.3, page 75. At this time point, on this occasion, the two assays show little correlation. It was decided that due to the non- sacrificial nature of

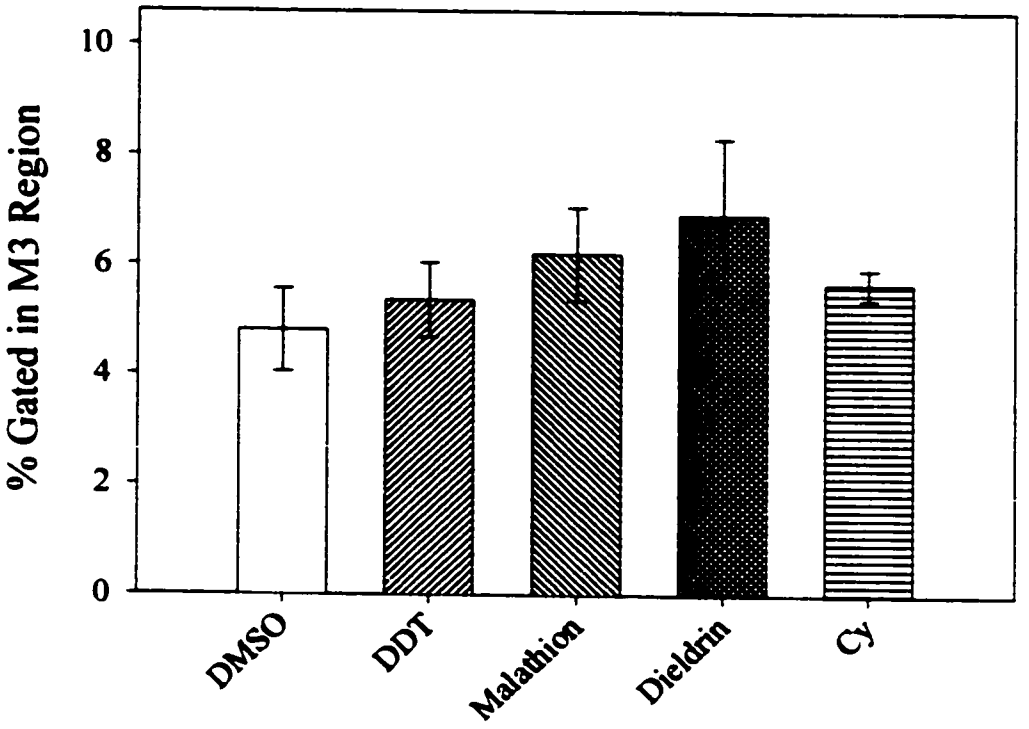

Figure A 7 – Flow cytometric analysis of M3 region, in leopard frog isolated spleen cells. M3 region indicates cells which have consumed three of more floresbrite microspheres. Each group n = 5. No statistical difference between groups as determined by one way ANOVA

the chemiluminescence (CL) assay, the small amount of blood required, its relative ease and the presence of the relevant instrumentation in
Windsor, that this was a more suitable assay than the phagocytosis for the rest of the study.

A.5.6.2.Phagocytosis of Cells Determined Using Whole Blood

In an attempt to make the phagocytosis assay non-sacrificial, the adherence of phagocytic cells from individual frog samples was undertaken. Blood was collected by cardiac puncture as previously described. The lymphocytes were removed over a histopaque 1077 gradient as described in section A.5.5.2. The remaining pellet was suspended in 5 mL of RPMI 1640 medium (JRH Bioscience, Lenexa, USA) supplemented with 10% FCS, 100 U penicillin, 100 μg/ml streptomycin and washed twice by centrifuging at 250 x G for 10 minutes. The pellet was resuspended in 1mL of medium, and aseptically pipetted into a 24 well plate to allow adherent cells to bind for 8 hours at 15°C. The plate was washed twice with Hanks balanced salt solution (HBSS), (JRH Bioscience, Lenexa, USA) to remove non-adherent cells. Trypsin-EDTA (500 μl) was used to remove adherent cells. The time taken for the cells to be removed was monitored by microscopy. When the cells had lifted 2 mLs of RPMI medium were added, the suspension was centrifuged at 250 x G for 2 minutes and the pellet resuspended in 0.5 mL of PBS. Cell viability was determined by diluting an aliquot of the cell suspension in 1:10 in 0.1% trypan blue and counting the number of viable macrophages by microscopy using a hemocytometer. The cell concentration was adjusted to 2×10^5 cells/mL and 0.5 ml of the cell suspension distributed in a 12 x 75mm

polypropylene tube. Flouresbrite carboxolate microspheres (1.716μ diameter, Polysciences Inc, Warrington, PA) were added at a ratio of 1:100 cell:beads. The suspension was incubated in the dark, at 26°C under light agitation for 18 hours.

Free beads were removed from the suspension using a gradient of 3% BSA and RPMI 1640 medium supplemented with 10% fetal calf serum. Acquisition was performed using a flow cytometer, 10,000 cells were analysed and results expressed as percentage of cells that phagocytosed 3 beads or more.

Fluorescent microscopy was used to determine that beads were taken up by the isolated phagocytic cells. Figure A8 shows an image of a phagocytic cell containing Floresbrite microspheres. This attempt to isolate phagocytic cells was partially successfully as some cells were found to be adhered to the plastic of the plate. These were successfully harvested and when viewed under a flourescent microscope it was clear that some cells were viable had consumed microspheres. Despite these successes for various reasons this assay was not considered reliable. These reasons included: difficulties in isolating enough cells from the blood sample of an individual frog and pooled samples were not considered as informative; and trouble lifting the cells from the plastic whilst still allowing them to remain viable and unfragmented. One frequently used method for isolating macrophages is to lyse the red blood cells. This could not be used in this case as the red blood cells of amphibians are nucleated and notoriously difficult to lyse.

A.5.7 Rapid Reversed Phase High-Performance Liquid Chromatography (HPLC) to Quantify Plasma Corticosterone

To ensure that corticosterone existed in measurable amounts in leopard frog

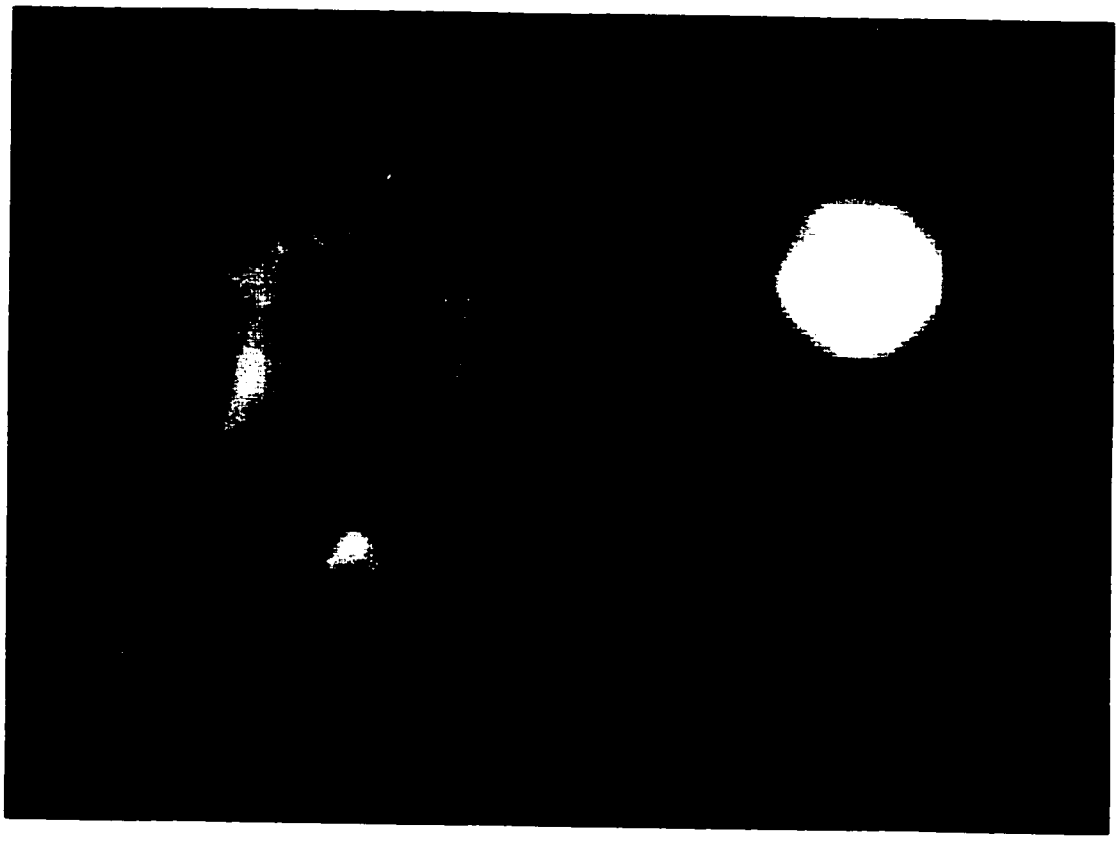

Figure A8 – Flourescent microscopy image of phagocytic cell next to a lymphocyte. Engulfed Flouresbrite microspheres can be seen within the phagocyte

plasma high-performance liquid chromatography (HPLC) was used. For the determination of multiple corticosterone samples radioimmunoassay (RIA) using a double labelled antibody I^{125} kit is more useful than this method as it is a rapid, accurate method which requires small quantities of blood. This method has successfully been applied for the measurement of corticosterone in other species such as gulis [127], mudpuppies [128]and turtles [129].

Extraction of frog plasma was undertaken as per the method set out in Woodward and Emery, 1987 [130]. Briefly, 1 mL of plasma was pipetted into screw capped culture tubes and 50µl of sodium hydroxide (0.3M) was added to prevent organic extraction of phenolic contaminants. Cortocosteriods were extracted into 5 mL diethyl ether-dichloromethane (60:40, v/v) by vortex mixing for 30 seconds and then centrifuged at 600 x g for 5 minutes with 1 mL of HPLC grade water. After recentrifugation, the supernatant (3 mL) was transferred to a reactivial and evaporated at room temperature under nitrogen. The residue was redissolved in 250µl of methanol-water (55:45, v/v).

Chromatograms for the standard steroids were obtained, the peak height plotted against the known standard concentration and an r^2 value obtained by linear regression. The r^2 was found to be 0.9334. Table A1 shows the peak height and the retention times of the corticosterone standards and a pooled frog plasma sample. The retention time of corticosterone fell between 3.9 and 4.3 minutes.

The chromatogram obtained from the pooled frog data indicated a peak at a retention time that would suggest the presence of corticosterone. This peak was however not clear as it was masked by a huge peak (674.7 mV) that was slightly earlier (RT 3.07 minutes). The origin of this larger peak is unknown but might be eliminated by

AMOUNT OF CORTICOSTERONE (μg/ml)	PEAK HEIGHT	RETENTION TIME (min)
5	252.9	3.992
2.5	62.62	4.194
1.25	26.63	4.254
0.5	16.16	4.337
Pooled Plasma Sample	27.54	3.803

Table A1 – Peak height and retention times of the corticosterone standards and the pooled plasma sample.

collecting relevant fractions, lypholysing it, then redissolving in solvent and rerunning this sample.

Plasma samples from rats showed a minor peak at about 0.5 min in front of corticosterone which the integrator processed separately (Woodward and Emery, 1987), this however does not explain the large size of the peak seen in this chromatogram.

Some problems were experienced when setting up the program. The isocratic nature of the program required some extra steps. Changes were made to incorporate a 10 minute equilibrium (55:45) run in addition to a 1 minute 100% methanol wash which was suggested in the published method. It is possible that 10 minutes was not a long enough period to wash the column and some unresolved compounds were still present.

This experiment was only done once, using one sample so more repeats are required in order to be sure that this extraction method and HPLC set up was correct for frog plasma. The original method was set up for rat plasma and there are important differences between mammalian and amphibian blood that might mean that alterations in the extraction procedure are required. The poor resolution of the corticosterone peak means that no useful conclusions can be drawn until fractionation and lypholisation of the sample have occurred. This would allow confirmation of the presence of corticosterone in the sample and eradication of the large unknown peak. The only draw back is the closeness of the two peaks as it might be difficult to ensure that a portion of the corticosterone in the sample might be collected in the previous fraction.
During this experiment recovery was not measured. This could have been achieved by spiking with an internal standard or by running duplicate samples with corticosterone added and measuring the increase in peak-area ratio although the results of these

techniques, as was used during the extraction method for rat plasma outlined by Woodward and Emery, 1987 [130]. They found that the recovery of the internal standard was 94.6±5.8% and the recovery of the corticosterone was 96.7±7.7%. The addition of spiked samples or duplicates to the experimental design would confirm the amount of corticosterone lost during extraction and injection phases.

It would also be useful to try other solvents and other mixtures for example acetonitrile in the place of methanol as this might improve the separation and resolution. The Woodward and Emery paper found that corticosterone was detectable in concentrations as low as 0.1 μg/mL. Although this is sensitive it is not comparable to the sensitivity of the RIA. The other advantage of the RIA is the sample size required. It is possible to obtain results with as little as 10 μl of plasma. This significantly smaller sample size would mean that it is not necessary to pool the frog samples. As the overall aim of the project is to track individual frogs from different areas the RIA is likely to be a superior method for routine analysis in this case. In conclusion corticosterone appeared to be present in the chromatogram of the pooled frog plasma sample although it was partially masked by the presence of a larger unknown peak.

A.6 CONCLUSIONS

Following these development stages three assays were used for the final studies. These were the antibody response to KLH-DNP which used the 6-16 antibody to measure specific IgM production, the delayed-type hypersensitivity reaction using PHA-P as the immunogenic injectable compound and the whole blood chemiluminescence (CL) assay. The assays for cell surface expression of class II MHC and phagocytosis of fluorescent

microspheres using spleen cells were both undertaken in the injection study, but due to inconclusive findings and problems with assay methodologies further development is being undertaken.

Appendix C - Organochlorines in Field Collected Leopard Frogs (ng/g w.w.)

	QCB	a-HCH	HCB	B-HCH	g-HCH	OCS	oxychlordane	transchlordane	cis-chlordane
Point Pelee 1	0.158	0.000	0.352	0.000	0.000	0.056	0.981	0.000	0.074
Point Pelee 2	0.000	0.000	0.186	0.000	0.000	0.076	1.017	0.000	0.100
Holiday Beach 1	0.100	0.000	0.251	0.000	0.000	0.000	107.329	0.167	6.694
Holiday Beach 2	0.000	0.000	0.115	0.000	0.000	0.072	0.855	0.000	0.071
Holiday Beach 2	0.000	0.000	0.150	0.000	0.077	0.171	0.829	0.000	0.172
Arner Townline 1	0.121	0.000	0.163	0.000	0.000	0.000	0.492	0.000	0.125
Arner Townline 1	0.131	0.000	0.092	0.000	0.000	0.000	0.660	0.000	0.039
Arner Townline 2	0.109	0.000	0.088	0.000	0.000	0.000	0.227	0.000	0.000
Perch Farm 1	0.107	0.000	0.235	0.000	0.000	0.000	0.310	0.000	0.000
Perch Farm 1	0.155	0.000	0.257	0.000	0.000	0.000	0.376	0.000	0.000
Perch Farm 2	0.144	0.000	0.170	0.000	0.000	0.042	0.341	0.000	0.137
Perch Farm 3	0.264	0.000	0.142	0.000	0.000	0.068	2.494	0.000	0.277
Collingwood 1	0.255	0.000	0.089	0.000	0.000	0.055	2.745	0.000	0.269
Collingwood 2	0.406	0.000	0.219	0.000	0.000	0.079	2.068	0.000	0.196
Collingwood 5	0.209	0.000	0.160	0.000	0.000	0.000	0.363	0.000	0.000
Ottawa 3	0.231	0.000	0.192	0.000	0.000	0.000	0.389	0.000	0.102
Ottawa 4	0.235	0.000	0.150	0.000	0.000	0.000	0.380	0.000	0.000
Ottawa 5	0.164	0.000	0.121	0.000	0.000	0.000	0.317	0.000	0.000

Appendix C - Continued

	trans nonachlor	pp'-DDE	p,p'-DDD	cis-noNDchlor	pp'-DDT	photomirex	mirex	heptachlor epoxide	dieldrin
Point Pelee 1	0.088	491.456	17.117	0.000	121.016	0.000	0.000	0.895	21.155
Point Pelee 2	0.067	16.895	0.200	0.000	2.936	0.000	0.000	0.655	28.288
Holiday Beach 1	16.792	5.394	0.000	1.633	0.511	0.000	0.000	8.508	0.564
Holiday Beach 2	0.069	6.436	0.158	0.000	0.954	0.000	0.000	0.757	3.304
Holiday Beach 2	0.255	7.565	0.522	0.059	1.066	0.000	0.000	0.789	3.407
Arner Townline 1	0.086	2.217	0.000	0.000	0.153	0.000	0.000	0.224	0.277
Arner Townline 1	0.000	2.915	0.000	0.000	0.819	0.000	0.000	0.241	0.345
Arner Townline 2	0.000	2.273	0.000	0.000	0.000	0.000	0.000	0.184	0.122
Perch Farm 1	0.000	1.006	0.000	0.000	0.255	0.000	0.000	0.298	0.173
Perch Farm 1	0.000	0.776	0.000	0.000	0.000	0.000	0.000	0.202	0.114
Perch Farm 2	0.196	1.092	0.000	0.000	0.000	0.000	0.000	0.242	0.197
Perch Farm 3	0.192	2.371	0.000	0.000	0.355	0.000	0.000	0.647	0.987
Collingwood 1	0.193	2.655	0.000	0.000	0.416	0.000	0.000	0.409	1.960
Collingwood 2	0.247	3.761	0.294	0.000	0.782	0.000	0.000	0.643	2.359
Collingwood 5	0.152	1.449	0.123	0.516	0.268	0.000	0.000	0.137	0.180
Ottawa 3	0.124	1.577	0.000	0.000	0.445	0.000	0.000	0.101	0.170
Ottawa 4	0.000	1.353	0.000	0.000	0.000	0.000	0.000	0.189	0.157
Ottawa 5	0.000	1.276	0.000	0.000	0.000	0.000	0.000	0.228	0.244

Appendix D - Concentrations of Polychlorinated Biphenyls in Field Collected Leopard Frogs (ng/g

	Concentration (ng/g) wet weight													
Frog Identificatio	sum PCB	PCB28	PCB31	PCB52	PCB49	PCB44	PCB42	PCB64	PCB74	PCB70	PCB66/95	PCB60	PCB101	PCB99
Point Pelee 1	14.58	0.000	0.000	0.164	0.000	0.000	0.000	0.000	0.151	0.085	0.000	0.000	0.388	0.530
Point Pelee 2	11.52	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.131	0.000	0.000	0.000	0.252	0.539
Holiday Beach 1	10.78	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.538	0.353	0.000	0.000	0.000	1.003
Holiday Beach 2	15.59	0.000	0.106	0.148	0.000	0.000	0.000	0.000	0.336	0.136	0.449	0.000	0.378	1.120
Holiday Beach 2	25.92	0.000	0.128	0.118	0.172	0.219	0.000	0.000	0.420	0.266	0.678	0.215	0.952	1.441
Arner Townline 1	0.987	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.123	0.000
Arner Townline 1	1.417	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.098	0.083
Arner Townline 2	5.048	0.000	0.000	0.288	0.084	0.108	0.000	0.000	0.000	0.113	0.319	0.097	0.373	0.208
Perch Farm 1	3.986	0.000	0.000	0.253	0.000	0.101	0.000	0.000	0.000	0.181	0.388	0.138	0.485	0.213
Perch Farm 1	2.494	0.000	0.000	0.155	0.000	0.073	0.000	0.000	0.000	0.000	0.000	0.000	0.290	0.529
Perch Farm 2	1.902	0.000	0.000	0.189	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.192	0.195
Perch Farm 3	2.300	0.000	0.000	0.091	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.192	0.137
Collingwood 1	3.523	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.159	0.134
Collingwood 2	5.019	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.227	0.185
Collingwood 5	2.396	0.000	0.000	0.141	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.137	0.126
Ottawa 3	2.246	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.163	0.138
Ottawa 4	1.366	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.103	0.109
Ottawa 5	0.771	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.087	0.078

Appendix D - Continued

	Concentration (ng/g) wet weight												
	PCB97	PCB87	PCB110	PCB151	PCB149	PCB118	PCB146	PCB153	PCB105	PCB141	PCB138	PCB158	PCB129
Point Pelee 1	0.000	0.000	0.098	0.000	0.131	0.872	0.338	2.747	0.000	0.000	1.519	0.000	0.159
Point Pelee 2	0.000	0.000	0.061	0.000	0.091	0.743	0.213	2.627	0.000	0.000	1.613	0.000	0.096
Holiday Beach 1	0.000	0.000	0.119	0.000	0.268	0.000	0.000	2.495	0.000	0.000	1.574	0.000	0.119
Holiday Beach 2	0.000	0.000	0.088	0.000	0.000	1.357	0.294	3.642	0.000	0.000	2.306	0.000	0.070
Holiday Beach 2	0.177	0.000	0.552	0.162	0.755	1.784	0.492	4.772	0.307	0.151	3.455	0.000	0.185
Arner Townline 1	0.000	0.000	0.000	0.000	0.000	0.161	0.000	0.201	0.161	0.000	0.166	0.000	0.000
Arner Townline 1	0.000	0.000	0.000	0.000	0.000	0.051	0.000	0.187	0.000	0.000	0.727	0.000	0.000
Arner Townline 2	0.080	0.000	0.231	0.094	0.273	0.287	0.046	0.443	0.621	0.000	0.777	0.000	0.000
Perch Farm 1	0.168	0.000	0.332	0.099	0.227	0.333	0.000	0.303	0.036	0.000	0.520	0.000	0.000
Perch Farm 1	0.000	0.000	0.070	0.000	0.000	0.082	0.000	0.148	0.404	0.000	0.618	0.000	0.000
Perch Farm 2	0.000	0.000	0.092	0.055	0.138	0.000	0.000	0.387	0.000	0.000	0.304	0.000	0.000
Perch Farm 3	0.000	0.000	0.125	0.000	0.109	0.216	0.000	0.346	0.038	0.000	0.235	0.000	0.000
Collingwood 1	0.000	0.000	0.131	0.051	0.127	0.231	0.000	0.605	0.290	0.204	0.299	0.000	0.000
Collingwood 2	0.000	0.000	0.162	0.063	0.161	0.353	0.080	0.797	0.027	0.247	0.538	0.000	0.062
Collingwood 5	0.000	0.000	0.146	0.000	0.109	0.187	0.000	0.344	0.231	0.137	0.229	0.000	0.000
Ottawa 3	0.000	0.000	0.157	0.000	0.109	0.251	0.000	0.367	0.000	0.218	0.280	0.000	0.000
Ottawa 4	0.000	0.000	0.000	0.000	0.000	0.198	0.000	0.317	0.178	0.000	0.193	0.000	0.000
Ottawa 5	0.000	0.000	0.000	0.000	0.000	0.098	0.000	0.157	0.000	0.088	0.132	0.000	0.000

Appendix D - Continued

	Concentration (ng/g) wet weight													
	PCB182/187	PCB183	PCB185	PCB174	PCB171	PCB200	PCB172	PCB180	PCB170/190	PCB201	PCB203	PCB195	PCB194	PCB206
Point Pelee 1	0.935	0.311	0.000	0.000	0.045	0.056	0.073	2.859	1.047	0.403	0.412	0.191	1.010	0.061
Point Pelee 2	0.669	0.358	0.000	0.000	0.000	0.000	0.045	2.139	0.800	0.230	0.301	0.086	0.494	0.036
Holiday Beach 1	0.707	0.280	0.000	0.000	0.000	0.000	0.047	1.729	0.604	0.199	0.218	0.000	0.481	0.047
Holiday Beach 2	0.729	0.432	0.000	0.000	0.000	0.000	0.032	2.204	0.818	0.132	0.272	0.077	0.436	0.029
Holiday Beach 2	1.350	0.686	0.064	0.213	0.090	0.070	0.081	3.052	1.247	0.353	0.445	0.196	0.625	0.049
Arner Townline 1	0.066	0.000	0.000	0.000	0.000	0.000	0.000	0.110	0.000	0.000	0.000	0.000	0.000	0.000
Arner Townline 1	0.198	0.000	0.000	0.000	0.000	0.000	0.000	0.072	0.000	0.000	0.000	0.000	0.000	0.000
Arner Townline 2	0.140	0.066	0.000	0.068	0.000	0.000	0.000	0.207	0.086	0.000	0.037	0.000	0.000	0.000
Perch Farm 1	0.093	0.000	0.000	0.000	0.000	0.000	0.000	0.115	0.000	0.000	0.000	0.000	0.000	0.000
Perch Farm 1	0.058	0.000	0.000	0.000	0.000	0.000	0.000	0.067	0.000	0.000	0.000	0.000	0.000	0.000
Perch Farm 2	0.181	0.072	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.057	0.041	0.000	0.000	0.000
Perch Farm 3	0.193	0.062	0.000	0.000	0.000	0.000	0.000	0.279	0.095	0.049	0.049	0.000	0.084	0.000
Collingwood 1	0.162	0.112	0.000	0.000	0.000	0.000	0.000	0.598	0.172	0.000	0.112	0.000	0.136	0.000
Collingwood 2	0.362	0.131	0.000	0.000	0.000	0.000	0.000	0.880	0.327	0.100	0.130	0.000	0.188	0.000
Collingwood 5	0.143	0.061	0.000	0.000	0.000	0.000	0.000	0.229	0.066	0.000	0.041	0.000	0.069	0.000
Ottawa 3	0.128	0.057	0.000	0.000	0.000	0.000	0.000	0.206	0.046	0.000	0.041	0.000	0.086	0.000
Ottawa 4	0.054	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.085	0.000	0.000	0.000	0.129	0.000
Ottawa 5	0.063	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.067	0.000

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